Vol. XLV No. 9

September 1956

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Editorial and Executive Offices—2215 Constitution Avenue, N.W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

Annual Subscription—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition and Scientific Edition combined: United States, Canada, Pan America, and Spain, $10.00; other foreign, $12.00.

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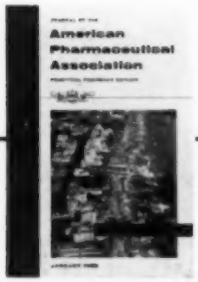

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLV — *SEPTEMBER 1956* — NUMBER 9
CONSECUTIVE NO. 18

The Use of Microscopic and X-ray Diffraction Methods for the Identification of Barbituric Acid Derivatives*

By W. G. PENPRASE† and JOHN A. BILES‡

Crystals of the barbituric acid derivatives were obtained by fusion, sublimation, crystallization from water, and crystallization from concentrated ammonia. The characteristic crystals allow a rapid means of identification of the pure crystalline derivative. X-ray diffraction powder diagrams were made and the *d*-distances reported. A discussion of the crystalline habits, phases and *d*-distances is presented.

IN THIS LABORATORY a rapid method of identification and differentiation of the barbituric acid derivatives has been developed using microscopic and X-ray procedures. This method is successful when only small amounts of sample are available. The study was made on powder, tablet, and capsule dosage forms.

Much has been published concerning qualitative methods of identifying the barbituric acid derivatives. Paulus (1) reported that the most reliable qualitative indications of the barbituric acid derivatives were obtained by measuring the physical constants and examining the microscopic appearance of the crystals. Lucas and McDougal (2) identified the barbituric acids by examining the crystals obtained by crystallizing from aqueous acid solutions. The optical-crystallographic properties of the barbiturates have been published (3–8).

Brandstätter (9) stated that microscopic identification was of little value because of isomorphism and polymorphism. He suggested the use of melting points and measurements of the refraction of the melts for purposes of identification. Microsublimation procedures have been suggested (10, 11). Gonzales, *et al.* (11), discuss in detail the different procedures of sublimation to identify pure compounds and to separate mixtures of solids.

Chromatographic methods (12, 13), polarographic determinations (14), nonaqueous acid-base titrations (15), ultraviolet spectra, (16, 17), and infrared spectra (18) have been reported as successful methods of identification of the barbiturates. Derivatives of the barbiturates have been made, and their melting points serve as a means of identification (19, 20).

X-ray diffraction studies have been published by Tso-Yueh Huang and co-workers, (21–23). These investigators have studied the powder diagrams of the pure barbiturates, different phases, mixtures, and eutectics of the barbituric

* Received April 9, 1956 from the pharmaceutical chemistry laboratories, School of Pharmacy, University of Southern California, Los Angeles 7, Calif.
† Police chemist, Los Angeles Police Department.
‡ Associate Professor, School of Pharmacy. Responsible for reprints.
This study was completed in part by a grant from the University of Southern California Pharmacy Alumni Association Development Program.
The authors wish to thank the following companies for supplying the compounds used in this study: Abbott (butethal, thiopental), Ciba (allobarbital), Hoffmann-La Roche (aprobarbital), McNeil (butabarbital), Massengill (alphenal), Parke, Davis (hexethal), Sandoz (allyl barbituric acid), Sharp and Dohme (vinbarbital), Winthrop-Stearns (cyclobarbital, hexobarbital, mephobarbital), and Upjohn (Cyclopal).

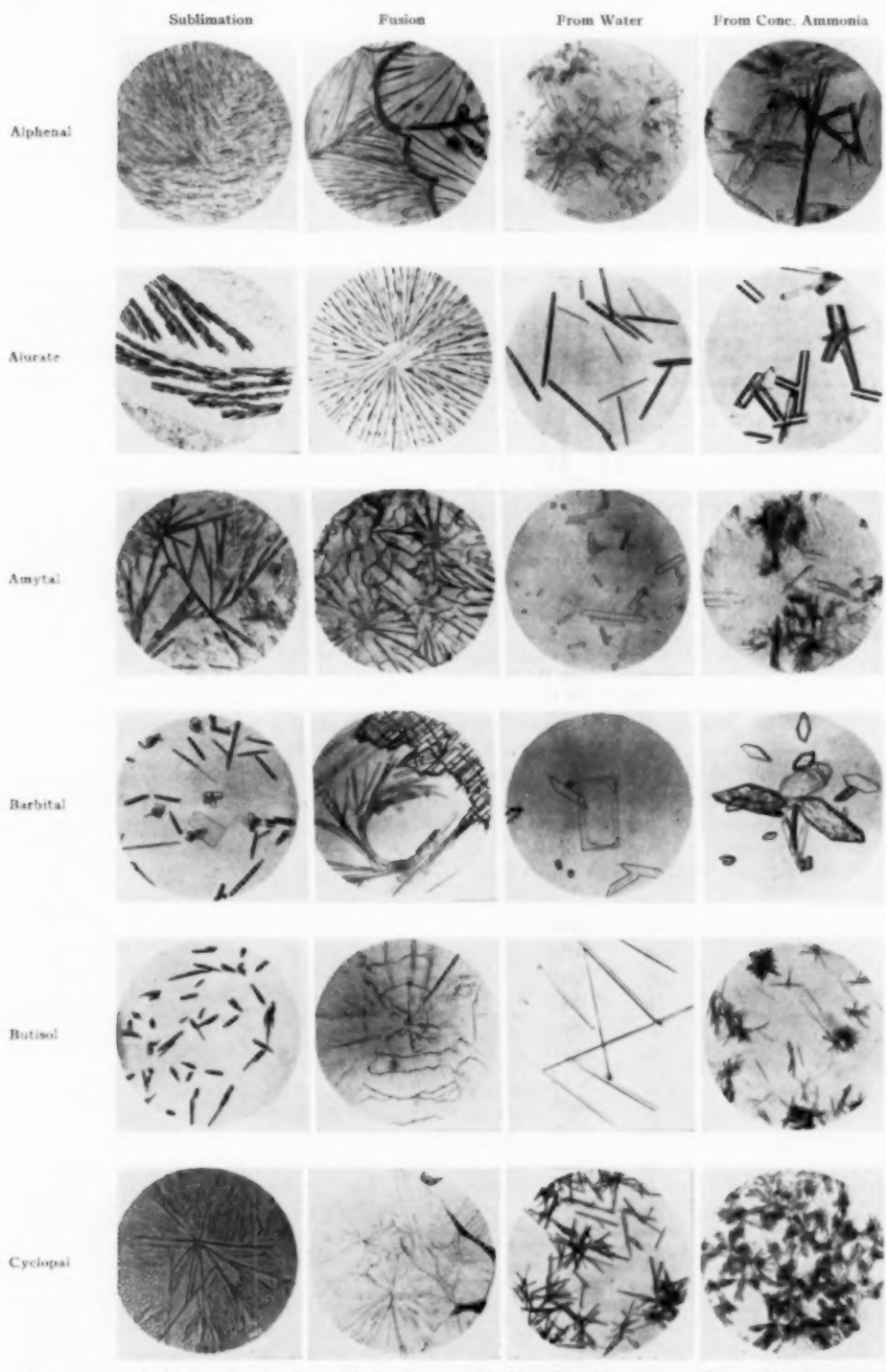
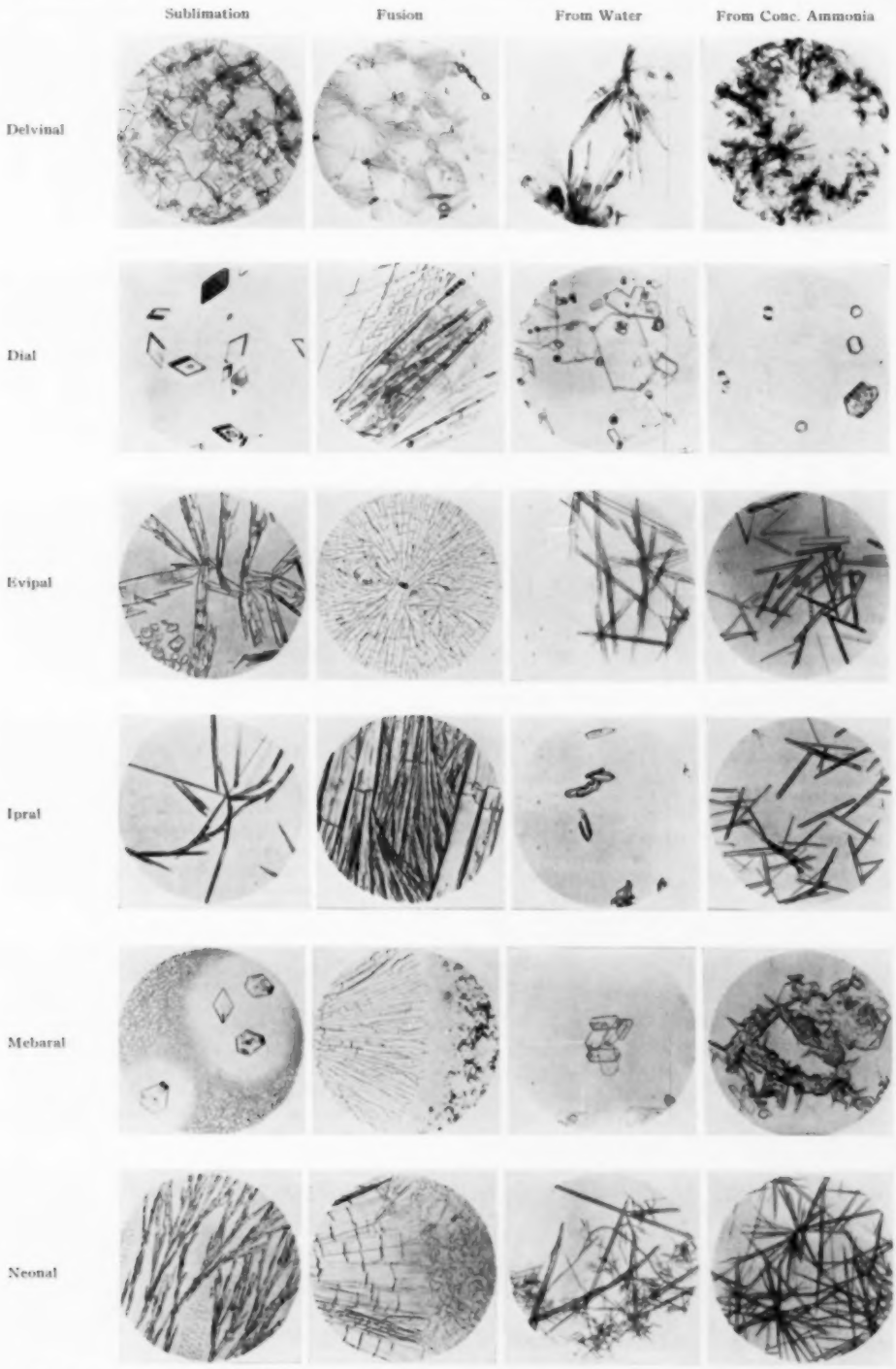


Fig. 1.—Photomicrographs showing variation in crystal habits of barbiturates. All magnifications ×45



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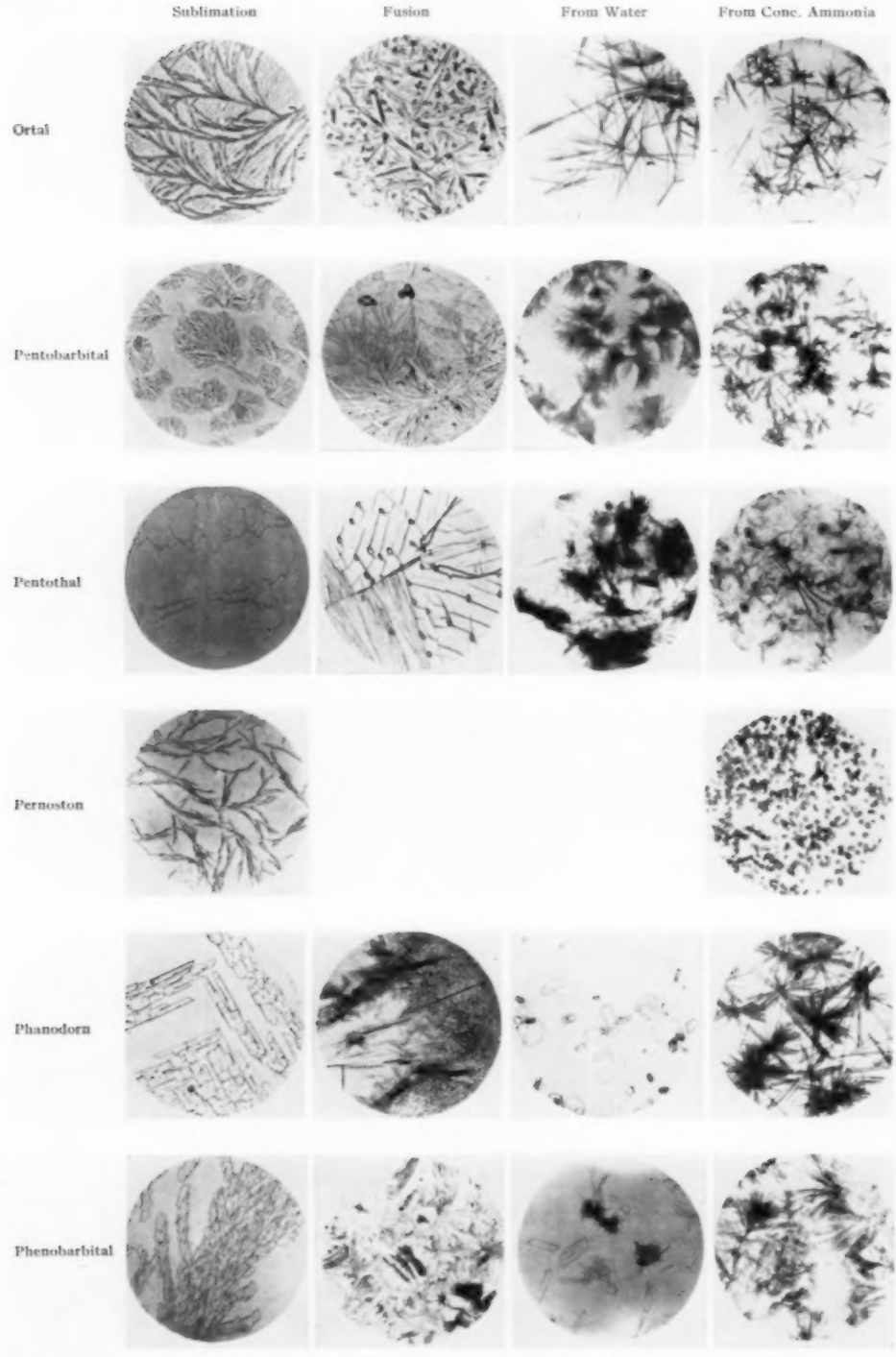

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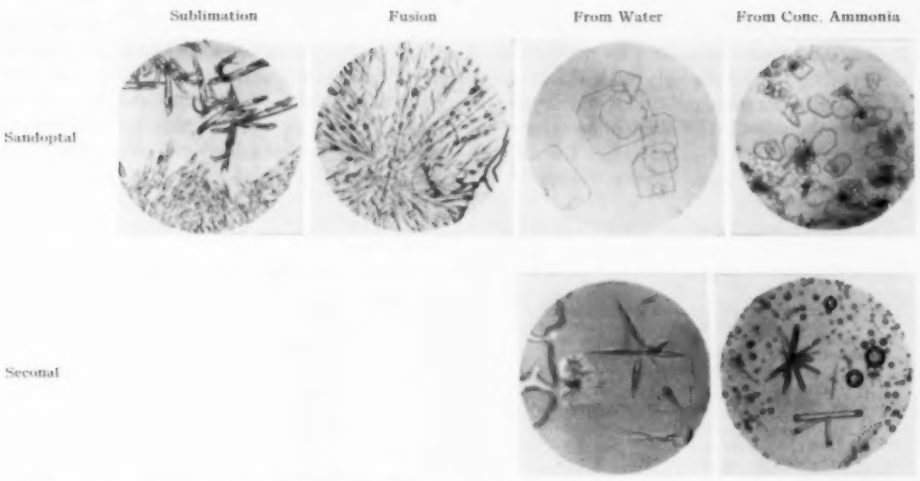

Fig. 1.—Photomicrographs showing variation in crystal habits of barbiturates. All magnifications ×45.

acids. A survey on the identification of barbiturates in forensic chemistry by X-ray diffraction methods was published in 1953 (24). The use of X-ray analyses for the identification of organic compounds in forensic chemistry has been discussed by Pinker (25) and Marvin and Garbarino (26).

EXPERIMENTAL

If the material under consideration gave a positive Koppanyi test, it was assumed that a barbituric acid might be present (27). The acid was solubilized in aqueous solution using dilute sodium hydroxide, and was filtered to eliminate the foreign material. The filtrate was acidified with dilute sulfuric acid. Sodium bicarbonate was added until the solution was slightly alkaline to prevent subsequent extraction of salicylates. The solution was then extracted three times with absolute ether. The combined ether solutions were washed and evaporated to dryness. The crystalline material after evaporation was recrystallized from a dilute hydroalcoholic solution.

Small portions of the crystallized barbituric acid derivatives were crystallized from water and concentrated ammonia. This procedure was performed on a microscope slide. Another small portion of the solid material was fused on a microscope slide. A fourth portion of the purified acid was placed on a hanging drop slide, covered with a cover slip and placed on the Kofler melting point apparatus. The temperature of the hot stage was increased and frequent examinations of the field were made. When there was an insufficient amount of barbituric acid derivative in the original extract to run each test, the small amount used for any one test was used again for each of the other procedures.

The melting points of the acids are published. In this laboratory the melting points of the barbituric acid derivatives were determined using the Kofler melting point apparatus.

For purposes of X-ray analysis the purified acid was finely ground (particle size to pass through a 200-mesh sieve) in an agate mortar and the resulting powder was mounted on a lead sample holder. The holder in turn was placed in position on the spectrometer's rotating specimen holder. The characteristic diffraction patterns were then obtained by subjecting the powder to Cu K-alpha radiation from the X-ray spectrometer and recording the diffracted radiation on a chart, using a modified GM tube with a recording potentiometer. The *d*-distances were calculated and are reported in Table I. The X-ray instrument used was a Norelco (North American Phillips Co.) Geiger-Counter X-Ray Spectrometer Type No. 12012, equipped with a recording potentiometer (Brown Electronik Strip Chart Pyrometer Single Point Recorder, High Speed), using a rotating specimen holder.

DISCUSSION

It was found that the temperature of sublimation of a pure barbituric acid derivative varied depending on the rate of temperature increase of the hot stage. Therefore, the temperature at which sublimation or vaporization begins is not characteristic unless the rate of temperature increase is standardized. With some of the acid derivatives sublimation does not occur. Instead condensation of a vapor phase of the acid occurs on the cover slip, and as the temperature continues to increase the condensate crystallizes. This phenomenon is particularly evident in the photomicrographs of the sublimation crystals of butethal, hexethal, and mephobarbital.

Generally speaking each pure barbituric acid derivative has a characteristic habit when crystallized from a particular solvent, such as water,

acetone, 80% alcohol, and concentrated ammonia. It is evident from the photomicrographs (Fig. 1) that the crystal habits vary with the choice of the solvent. In most conditions, the crystalline phase is the same. The ammonium salts of the barbiturates were not isolated when the acid was crystallized from concentrated ammonia.

days of standing. Crystals from concentrated ammonia were obtained after four hours of standing. Sublimation and fusion methods were likewise unsatisfactory. Seeding the secobarbital was of no value. Very satisfactory crystals of butallylonal were obtained when crystallized from concentrated ammonia. No characteristic crys-

TABLE I.—THE *d*-DISTANCES FOR THE PURIFIED BARBITURIC ACID DERIVATIVES USING CU K-ALPHA RADIATION

Alphenal, *d*-Distance	Amobarbital Amytal, *d*-Distance	Aprobarbital Alurate, *d*-Distance	Barbital, *d*-Distance	Butabarbital Butisol, *d*-Distance	Cyclopal, *d*-Distance	Vinbarbital Delvinal, *d*-Distance
13.35	10.50*	6.64*	7.06	9.80*		13.56*
6.74*	7.00	6.49*	6.37	6.66*	9.19*	6.84
5.63	6.05	6.09	5.70	5.52*	7.12	6.13*
4.81	5.49*	5.89	3.89	5.31	6.22*	4.97
4.49*	5.36*	5.52	3.68	5.00	5.89	4.86*
4.26	4.86	5.11*	3.54*	4.81	5.66	4.61
3.94	4.47	4.49	3.34*	3.76	5.49	4.22
3.37*	4.03	3.81	3.04*	3.68	4.97	3.47
3.06	3.79	3.55	2.97	3.35	3.89	3.20
2.69	3.59	3.37	2.66	3.00	3.35*	3.13
		3.27			3.26	

Allobarbital Dial, *d*-Distance	Hexobarbital Evipal, *d*-Distance	Probarbital Ipral, *d*-Distance	Mephobarbital Mebaral, *d*-Distance	Butethal Neonal, *d*-Distance	Hexethal Ortal, *d*-Distance	Pentobarbital Nembutal, *d*-Distance
6.40*	10.43*	9.17*	12.78*	10.37*	11.02*	11.02*
6.01	8.02	8.73*	6.49*	9.80*	10.14	7.88
5.66	5.63*	6.26	5.97	7.26	7.74	6.98*
5.39	5.29	5.52	5.32	6.92*	7.35	5.52*
5.26	4.26	5.08	4.83	5.36	5.11*	4.81
5.02*	4.14	4.36*	4.12	4.87	4.81	4.47
4.86	3.89*	4.10	3.76*	4.43	4.36*	4.01
4.01	3.67	3.60	3.58	3.98	3.94	3.76
3.64	3.18	3.35	3.18	3.70	3.34	3.55
3.25*	2.61	3.14	2.99	3.18	3.20	3.03
						2.73

Thiopental Pentothal, *d*-Distance	Butallylonal Pernoston, *d*-Distance	Cyclobarbital Phanodorn, *d*-Distance	Phenobarbital, *d*-Distance	Allyl Barbituric Acid Sandoptal, *d*-Distance	Secobarbital Seconal, *d*-Distance
12.59*	8.09	11.02*	11.60	10.50*	10.02
6.74*	7.06	7.12	6.38*	10.31	7.54
6.18	6.69	6.13*	5.77*	6.79	7.18
5.02	6.35*	5.56	5.34*	6.49*	6.45*
4.63*	5.93	5.39	5.01	5.70	6.13*
4.12	5.20	5.17	4.78	5.36*	5.36*
3.81	4.20	4.81	4.47	5.00	5.02
3.62	3.86*	4.40	4.03	4.10	4.16
3.38	3.39*	3.55	3.57	3.58	3.55
3.26		3.28	3.21	3.46	3.07

* Strong.

Characteristic habits were obtained when the barbituric acid was fused and sublimed. Therefore, the procedure of crystallization from different solvents coupled with fusion and sublimation forms the basis for the rapid identification of the barbituric acid derivatives.

This procedure is not satisfactory for the rapid identification of secobarbital. Suitable crystals of secobarbital were obtained from water only after tals were obtained from water, and none were obtained with fusion.

Observation of the crystals, particularly the fused material, between crossed nicols was of value during microscopic examination. Additional proof for identification is available through examination of interference figures. Partial or complete determination of optical-crystallographic data is valuable.

For those unfamiliar with X-ray diffraction, it should be pointed out that the patterns obtained from a recording X-Ray Spectrometer consist of a series of peaks of varying intensity. The patterns are a result of the diffraction of essentially monochromatic radiation from a crystalline material acting as a diffraction grating. When properly prepared and when subjected to electromagnetic radiation of an appropriate wavelength, a crystalline material acts as a three-dimensional diffraction grating because of its unique combinations of appropriately placed periodically arranged particles.

For our purpose, Cu K-alpha radiation was used. When the crystalline material is powder, mounted and rotated through a suitable number of degrees, diffraction of the radiation at characteristic angles and intensities occur. An arc 50 degrees of traverse on the goniometer arm is sufficient for barbituric acid derivatives. The peaks of varying intensity occur at twice the angle (2θ) of diffraction. From these values (2θ), the angles of diffraction (θ) are determined. Knowing the angle of diffraction and the wavelength of the radiation (1.53736 Å.) (28), the *d*-distances can be calculated using Bragg's law; or these values can be obtained from tables (28). The reproducibility of the angles at which the peaks occur for the same barbituric acid derivative on various runs, as here reported, is of the order of ± 0.1 degree.

The procedure followed in this laboratory in the identification of the barbituric acid derivatives by X-ray diffraction patterns is to directly compare the pattern of a suspected material with that of a standard. Agreement in the overall pattern of the two constitutes an identification. If the peaks (2θ values) of the unknown pattern and peaks of the standard pattern agree as to angle and intensity, the material present in the standard is present in the unknown. (NOTE: There may be present in the powder under investigation other material than that shown in the pattern.) Characteristic of the crystalline barbituric acid derivatives under standard conditions is the consistent reproducibility of the angles of diffraction and their respective intensities.

The use of the crystal form by the methods discussed and the use of *d*-distances have been valuable to the senior author for the identification of barbituric acid derivatives. Working in the Criminalistics Laboratory of the Los Angeles Police Department, the senior author has rapidly identified specific barbituric acid derivatives and has found the characteristic crystals obtained to be extremely reliable. It is essential, for consistently reproducible results, that the barbituric acid derivative be pure and that the methods of extraction and purification be standardized.

Work is being continued with the newer barbituric acid derivatives, the hydantoins, and the nonbarbiturate sedatives. This work will be the subject of a later paper.

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The Anticurare Activity of Two Alkyl Phosphates*

By B. LEVY,† T. S. MIYA, and L. D. EDWARDS‡

The anticurare activities of tetraethylpyrophosphate (TEPP) and parathion were compared with those of neostigmine methyl sulfate (Prostigmin®). Two methods were employed involving the rat and the rabbit. In the first procedure, using the rat phrenic nerve-diaphragm, TEPP appeared to be more effective than Prostigmin which was in turn more effective than parathion. In the second procedure, however, which involved pretreating the rat phrenic nerve-diaphragm, none of the three antagonists differed significantly. Prostigmin appeared to be more effective than the other two antagonists in raising the headdrop dose of Intocostrin® in the rabbit, but only at the lower dosage level. As the dosage for all antagonists was raised, their action did not differ significantly.

THE USE OF curare in clinical medicine is of recent origin. In 1935, King identified the active principle of this drug as being a quaternary base, *d*-tubocurarine. Until then, curare had not been used to any extent clinically because of its variable composition.

With the availability of a standardized preparation, the clinical use of curare increased rapidly. Up to the present time the most promising field of usefulness for curare has been as an adjuvant in anesthesia for the attainment of more complete muscular relaxation without the use of excessively deep anesthesia.

The clinical use of curare is often accompanied by certain toxic manifestations (1), the most serious being respiratory arrest by diaphragm paralysis. Other complications often encountered are salivation, bronchospasms, and laryngospasms. With serious overdosage of curare, artificial respiration by external pressure is of no avail because the muscular tone has been lost. Other toxic signs accompanying curare administration are persistent diplopia, dysarthria, or dysphagia (2). While it is true that the occurrence of respiratory failure will usually demand positive pressure artificial respiration, the length of time it must be continued can be greatly reduced by a suitable curare antagonist.

Much work has been done toward the development of a suitable anticurare agent which will be effective against severe curare overdosage. Most of this work, with a few exceptions, has been done on the Prostigmin homologs (3–6).

The objective of this study was to determine the anticurare activity of the two condensed alkyl phosphates; tetraethylpyrophosphate (TEPP) and diethyl-*p*-nitrophenyl thiophosphate (parathion). These two compounds have been shown to be very potent inactivators of cholinesterase. Since the principal actions of neostigmine methyl sulfate (Prostigmin) are mediated through its inactivation of cholinesterase, it was thought that TEPP and parathion might exert a more powerful anticurare action than Prostigmin.

EXPERIMENTAL

Rat Phrenic Nerve Diaphragm.—The anticurare activities of TEPP, Prostigmin, and parathion were compared on the rat phrenic nerve-diaphragm preparation (7) in two ways. In both procedures, the concentrations of all three antagonists were the same. TEPP and parathion were available in the form of stock solutions of the drugs in dry propylene glycol from which aqueous dilutions of 1:100,000 were prepared just prior to use. Prostigmin was also prepared in an aqueous dilution of 1:100,000. An aqueous Intocostrin solution was used in a concentration that contained two curare units per cc.

In the first procedure, equal doses of Prostigmin and one alkyl phosphate were compared on the same preparation. Each antagonist was added to the bath one minute before the addition of varying doses of Intocostrin. The dosage range of Intocostrin varied between 0.4–2.5 μg./75 cc. bath.

TABLE I.—RATIOS OF THE ACTIVITIES OF TEPP, PARATHION, AND PROSTIGMIN* (PROSTIGMIN = 1)

Concn. of Antagonist (μg./75 cc.)	TEPP	Prostigmin	Parathion
1	1.17	1	0.85
2	1.35	1	0.83
3	1.35	1	0.69
4	1.21	1	0.74
5	1.59	1	0.82
Mean	1.33	1	0.78
S.E.	±0.05		±0.03
t value TEPP + Prostig.	6.60		
t value parath. + Prostig.	7.33		
t value TEPP + parath.	9.43		

* The ratios of the activities of the three compounds were based upon their comparative ability to increase the dose of Intocostrin needed to produce 20% inhibition of the contraction of the rat diaphragm muscle.

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The Intocostrin was then allowed to act for two minutes. At the end of this period the bath was flushed and refilled with fresh Tyrode's solution. Ten minutes was allowed between tests during which time the preparation was washed four times. With this procedure, the preparation usually returned to its normal height of contraction. By plotting the per cent inhibition of contractions of the diaphragm muscle against the dosage of Intocostrin, the amount of Intocostrin needed to produce 20% inhibition of the contractions of the diaphragm could be read directly from the curve. The results are shown in Table I.

Rapid intravenous injections were employed for the antagonists studied. They were injected into the marginal ear vein one minute before the administration of the curarizing agent.

The crossover technique was used in the studies of the effective dosage range of Prostigmin, parathion, and TEPP. Since Prostigmin has been found to be very effective in a dosage range of 0.0125 to 0.075 mg./Kg. this range was employed for all antagonists.

A group of four rabbits was used for all of the tests. Four control mean head-drop doses were obtained on four consecutive days after first giving each

TABLE II.—COMPARATIVE ABILITY OF TEPP AND PROSTIGMIN TO BLOCK THE INHIBITORY EFFECTS OF INTOCOSTRIN ON THE RAT-PHRENIC NERVE DIAPHRAGM (PROSTIGMIN = 1)

	Antagonist Added 0 Minutes before Intocostrin	Antagonist Added 3 Minutes before Intocostrin	Antagonist Added 6 Minutes before Intocostrin
	1.28	1.16	..
	1.07	1.22	0.97
	1.11	0.89	0.79
	0.89	0.83	0.69
	1.08	0.94	..
	0.85	0.77	1.29
Mean	1.05 ± S.D.0.14	0.97 ± S.D.0.17	0.94 ± S.D.0.23
	± S.E.0.07	± S.E.0.07	± S.E.0.11
t value =	0.71	.43	.55

TABLE III.—COMPARATIVE ABILITY OF PARATHION AND PROSTIGMIN TO BLOCK THE INHIBITORY EFFECTS OF INTOCOSTRIN ON THE RAT-PHRENIC NERVE DIAPHRAGM (PROSTIGMIN = 1)

	Antagonist Added 0 Minutes before Intocostrin	Antagonist Added 3 Minutes before Intocostrin	Antagonist Added 6 Minutes before Intocostrin
	1.11	0.92	0.58
	0.93	1.37	1.07
	0.92	0.91	0.76
	0.81	1.04	0.87
	0.92	0.57	0.51
	0.99	0.56	0.83
Mean	0.95 ± S.D.0.09	0.90 ± S.D.0.28	0.77 ± S.D.0.34
	± S.E.0.04	± S.E.0.12	± S.E.0.08
t value =	1.25	0.83	2.62

In the second procedure, equal doses of Prostigmin and one alkyl phosphate were compared on the same preparation. The antagonist was added to the bath three minutes and six minutes prior to a constant dose of Intocostrin. The Intocostrin was allowed to act for 3 minutes. The bath was then flushed and refilled with Tyrode's solution four times over a ten-minute period. The dose of all antagonists was 1 μg./75 cc. bath. The results are presented in Tables II and III.

Rabbit Head Drop.—In this test parathion and TEPP were used in a dilution of 1:4000. These two aqueous dilutions were freshly prepared from a 5% stock solution of these drugs in propylene glycol. Prostigmin was also prepared in aqueous dilution of 1:4000. The potency of the dilution of Intocostrin was such that 1 cc. of the dilution contained four curare units.

The animals were unrestrained. Injections were made into the marginal ear vein at a rate of 0.05 cc. of Intocostrin solution every twenty seconds. animal a priming dose of curare. Following this, the mean head-drop dose of the group was obtained after pretreatment with a dose of TEPP. This procedure was repeated on consecutive days with the same dose of Prostigmin and parathion. This was the procedure used in comparing all of the doses of the antagonists studied.

The results of each assay were averaged and were subjected to statistical analysis, using methods for correlated data in comparing the head-drop dose following antidotal agents with the control head-drop doses in the same animals. The results are presented in Tables IV and V.

DISCUSSION

In this investigation the anticurare activities of TEPP and parathion have been compared quantitatively to that of Prostigmin in the rat and rabbit.

TEPP and parathion have been found to be very potent inactivators of cholinesterase. Since the

TABLE IV.—CONTROL HEAD-DROP DOSES ON FOUR CONSECUTIVE DAYS (UNITS PER KG.)

Rabbit No.	1st day	2nd day	3rd day	4th day
1	1.27	1.18	1.11	1.11
2	1.47	1.41	1.15	1.10
3	1.25	1.26	1.37	1.51
4	1.12	1.12	1.22	1.26
Mean	1.27	1.24	1.21	1.24
Stand. dev.	0.14	0.13	0.11	0.60

TABLE V.—HEAD-DROP DOSES OF INTOCOSTRIN FOLLOWING PRETREATMENT WITH TEPP, PROSTIGMIN, AND PARATHION (UNITS/KG.)

	Dose of Curare Antagonist, 0.0125 mg./Kg.			Dose of Curare Antagonist, 0.025 mg./Kg.			Dose of Curare Antagonist, 0.05 mg./Kg.		
	TEPP	Prostigmin	Parathion	TEPP	Prostigmin	Parathion	TEPP	Prostigmin	Parathion
	1.18	2.23	1.31	1.45	1.79	1.41	1.63	2.33	1.48
	1.38	1.95	1.46	1.62	2.26	1.61	2.11	2.01	1.68
	1.27	1.81	1.47	1.47	1.87	1.71	1.72	1.86	2.35
	1.21	1.57	1.11	1.89	1.96	1.25	1.95	1.80	1.28
Mean	1.26	1.89	1.34 (.71)	1.61	1.97	1.50 (.76)	1.85	2.00	1.70
S.D.	±0.09	±0.28	±0.17	+0.2	±0.21	±0.21	±0.22	±0.24	±0.46
S.E.	±0.04	±0.14	±0.08	±0.10	±0.11	±0.10	±0.11	±0.12	±0.23
t value Tepp + Prostig. =			4.33			2.42			0.92
TEPP + parath. =			0.89			0.77			0.59
parath. + Prostig. =			3.41			3.16			1.16

principal actions of Prostigmin are mediated through its inactivation of cholinesterase, it was thought that TEPP and parathion might exert a more powerful anticurare action than Prostigmin. This observation may be criticized on the basis of the fact that Prostigmin probably also exerts a direct musculotropic effect upon striated muscle. At the time that this investigation was carried out, Prostigmin was the most widely used curare antagonist. Edrophonium had not yet been made available for investigational purposes.

TEPP and parathion both exhibited anticurare activity. Two different dosage procedures were employed with the rat phrenic nerve diaphragm preparation. In the first procedure the order of effectiveness was as follows; TEPP, Prostigmin, and parathion. In the second procedure none of the three antagonists differed significantly from each other. The rabbit head-drop test showed Prostigmin to be more effective than either of the other antagonists in a dosage of 0.0125 mg./Kg. At a dosage of 0.025 mg./Kg. Prostigmin was more effective than parathion but not TEPP. Finally at a dosage of 0.05 mg./Kg. Prostigmin did not differ significantly from either TEPP or parathion. The results obtained with the rabbit head-drop method would appear to be more valid than those obtained with the rat phrenic nerve diaphragm since it is an *in vitro* test. Because of toxic symptoms the dosage of the antagonists was no higher than 0.05 mg./Kg.

SUMMARY

1. The anticurare activities of the two condensed alkyl phosphates, tetraethylpyrophosphate (TEPP) and parathion, have been compared quantitatively to that of neostigmine methylsulfate (Prostigmin). Two animal preparations were used in this study.

2. On the rat phrenic nerve diaphragm preparation, the ratios of the anticurare activities for the three agents were found to be as follows: 1.33, 1, and 0.78 for TEPP, Prostigmin, and parathion, respectively. All of these activity ratios were significantly different. In the second procedure involving the use of the rat phrenic diaphragm preparation all three compounds exhibited anticurare activities but none differed from the other significantly.

3. The rabbit head-drop method was used to determine the comparative ability of all three antagonists to raise the level of the mean head-drop dose of Intocostrin. In a dosage of 0.0125 mg./Kg. Prostigmin raised the head-drop dose of Intocostrin to a greater extent than did either of the other two agents. At a dosage of 0.025 mg./Kg. Prostigmin was more effective than parathion but did not differ significantly from TEPP. Finally at a dosage of 0.05 mg./Kg. Prostigmin did not differ significantly from either TEPP or parathion.

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Investigation of *Orthosiphon pallidus* Royle I*

Preliminary Chemical Study and Isolation of "Orthosiphonol"

By N. K. BASU and HARKISHAN SINGH

Preliminary chemical study of *Orthosiphon pallidus* Royle has been made. Analyses of ash and determination of extractives with various solvents have been done. The plant shows the presence of some alkaloidal bodies. From the unsaponifiable matter of the petroleum ether extractive, a pure white crystalline substance, provisionally named as "orthosiphonol" has been isolated. Its molecular formula has been found to be $C_{30}H_{50}O_2$. The infrared spectra of this compound and its acetate have been studied.

INNUMERABLE DRUG PLANTS in the Indian materia medica of old are described in Sanskrit literature. Quite a large number of vegetable remedies, however, are not mentioned in such literature although they have been used in the treatment of diseases. They have only been handed over from person to person and have thus been carried down through the ages. Rupmanjari, the drug plant under investigation, also known as Devamanjari, or Churka, is a drug belonging to this category. This drug, which is reputed for its efficacy in the treatment of neurasthenia and considered to be a general tonic and aphrodisiac, was introduced to one of the authors (N. K. Basu) by the late Dr. J. N. Gupta of Banaras, with a suggestion to carry out investigations regarding its chemical constituents and medicinal properties.

The plant was collected in its full bloom and identified as belonging to the *Labiatae* family, and then spotted out to be *Orthosiphon pallidus* Royle, by Professor N. K. Tewari of the Botany Department, Banaras Hindu University. It is distributed throughout the greater part of India from Kashmir and the Punjab to west Bihar and southwards to Travancore. It also grows in Beluchistan and Arabia (1, 2).

A survey of the literature revealed no report of a chemical or pharmacological study of the plant. In 1948, Basu and Mithal (3), were able to isolate a water-soluble alkaloid (m. p. 112–113°) from an aqueous extract of the plant. The quantity isolated being very minute, no systematic chemical work on the alkaloid was done. They were also able to isolate a steam-volatile principle, m. p. 52–53°. The pharmacognostical characters were also studied by the same authors.

The present authors started a systematic inquiry into the constituents of this drug and a study of its pharmacological activity.

* Received February 27, 1956, from the Department of Pharmaceutics, Banaras Hindu University, Banaras, India.

EXPERIMENTAL

Preparation for Analysis.—The drug was collected locally in the month of September, 1952, and identified as *Orthosiphon pallidus* Royle. The whole plants were dried, pulverized to moderately coarse powder, and used for the subsequent analysis.

Determination and Analysis of Ash.—The methods used for the determination of ash content were those of the British Pharmacopoeia 1953 (4). The results are as follows: Total ash, 21.76%; water-insoluble ash, 19.54%; water-soluble ash (by difference), 2.22%; acid-insoluble ash, 13.00%; and acid-soluble ash (by difference), 8.76%.

The total ash was analyzed qualitatively using a semimicro technique. The elements found to be present are: iron, calcium, potassium, zinc, chlorine as chloride, phosphorus as phosphate, and silicon.

Alkaloids and Glycosides.—For testing the presence of alkaloids and glycosides the Stas-Otto method (5) was employed. The drug did not show any positive indication for the occurrence of chloroform-soluble alkaloids or glycosides. Some water-soluble bases were found to be present, as seen by the positive response of the final aqueous layer to Dragendorff's reagent and saturated ammonium reineckate solution.

Extractives.—One hundred grams of the drug powder was extracted successively with the solvents mentioned in the order given below, employing a Soxhlet continuous extraction apparatus, the preceding solvent being completely removed from the marc before starting extraction with the next solvent. The extraction with water was carried out by digesting the marc with it on a steam bath. The values of per cent extractives obtained are as follows: Petroleum ether, 2.43%; benzene, 1.39%; ether, 0.38%; chloroform, 0.68%; acetone, 0.99%; alcohol (95% v/v), 4.14%; and water, 7.12%.

It was observed that the petroleum ether extract deposited crystals embedded in dark green resinous matter. From the alcoholic extract a brown granular deposit also separated, which was termed as "Alcoholic Fraction." This granular deposit later did not dissolve in 95% alcohol, but readily dissolved in water.

It was found that chloroform extracted substances which reduced Fehling's solution, whereas acetone, alcohol, and water, in addition to such reducing substances also extracted glycosides or disaccharides. It was also observed that the alcohol

and water extracts gave positive tests for alkaloids with Dragendorff's reagent and saturated ammonium reineckate solution, the extraction of alkaloids being more prominent with alcohol. The other extracts did not show the presence of any alkaloids.

The different extracts in their concentrated form did not, even on prolonged refrigeration, yield anything crystalline, besides the deposits formed in the case of petroleum ether and alcohol extracts.

Alcoholic Fraction.—The "Alcoholic Fraction" was found to consist of a mixture of reducing sugars, and disaccharides or glycosides. It was, however, reserved for its pharmacological screening before undertaking further chemical elucidation.

Petroleum Ether Extract.—Eight hundred grams of the drug, in moderately coarse powder, was extracted with petroleum ether (b. p. 60–80°) by hot continuous extraction for thirty hours. Upon concentrating the extract some crystals started depositing on the sides of the vessel along with a quantity of muddy, greenish matter. The crystals could not be freed from the greenish matter by washing with petroleum ether.

Separation of Unsaponifiable Portion.—The petroleum ether extract was mixed with its counterpart obtained during preliminary work and the solvent removed. The residue was found to be a treacle-like mass, which solidified on cooling. It was saponified by refluxing with 200 ml. of 0.5 *N* alcoholic potassium hydroxide for one and one-half hours. The major portion of alcohol was removed by distillation under reduced pressure, the concentrate was diluted with water, and extracted repeatedly with ether. The mixed ether extract was washed free of alkali with water. The ether was removed and the concentrate was reduced to about 15 ml., an equal volume of acetone was added and dried by keeping under vacuum. The unsaponifiable residue weighed 7.78 Gm. Some colorless needle-shaped crystals were found emerging from the residue.

Isolation of a Crystalline Substance.—The unsaponifiable residue was refluxed repeatedly with absolute alcohol but much of the brown resinous matter remained undissolved. The mixed alcoholic extract was concentrated to about 15 ml. and cooled. On cooling the concentrate formed a loose translucent mass. The latter was filtered by suction. The residue on the filter was waxy and brown in color. After removing much of the coloring matter by washing with ether the waxy residue (about 10 mg.) melted at 72°.

The alcoholic filtrate on cooling did not form a translucent mass, indicating that the setting property of the concentrate was due to the residue removed by filtration. The filtrate was concentrated and kept in a refrigerator for a few days, where some crystals started separating. The crop was purified by repeated crystallization from absolute alcohol until the melting point of the final crop was constant. The pure substance melted sharply at 143–145°. It weighed 73.4 mg. (0.0082% of the air-dried drug). *Anal.*—Calcd. for $C_{30}H_{50}O_2$: C, 81.84; H, 11.26. Found: C, 81.70; H, 11.17. Mol. wt., 422.5 (by cryoscopic method with benzene as solvent). Calcd. for $C_{30}H_{50}O_2$. Mol. wt., 442.

Color Tests.—Liebermann's test (6) produced a violet coloration, which instantly changed to blue, and finally to green.

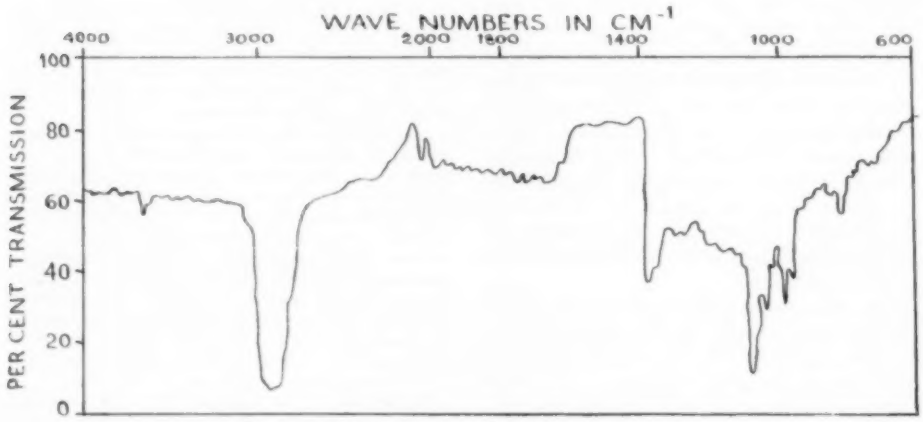

Fig. 1.—Infrared spectrum of the isolated substance.

On performing Hesse's test (6) the zone of contact of chloroform and concd. sulfuric acid became red in color, the lower layer being orange and the upper one dirty brown in color. It may be noted that the mixture manifested a green fluorescence, which was more prominent at the zone of contact and in the upper chloroform layer.

In Moleschott's test (6), small particles of the substance, on being warmed with a mixture of sulfuric acid and water (5:1), attained a distinct violet coloration. In Mach's test (6) a bright red scaly residue was left behind, which even after two to three washings with water, did not lose its metallic luster. Hirschsohn's test (6) was inconclusive.

Derivatives.—To prepare the digitonide, 5 mg. of the substance was dissolved in 5 ml. of 95% alcohol by heating and to the hot solution was added 2 ml.

of 0.5% digitonin solution in 95% alcohol. There was no immediate precipitation, but on warming, precipitation took place; warming was continued for five minutes to complete the reaction. The precipitate was filtered and washed twice with 4–5 ml. of warm 95% alcohol. The residue on the filter paper was further washed five times with peroxide-free ether. The digitonide so obtained was dried under vacuum. On determination of its melting point the substance changed to a semi-transparent melted mass at 202–205°, and slowly started charring when heated above this temperature.

The acetate was prepared, using 3–4 mg. of the substance, from acetic anhydride and purified pyridine system. The derivative shrank at 118° and melted at 124–125°.

Infrared Absorption Spectra.—The infrared absorption spectra of the isolated crystalline substance (Fig. 1) and its acetate (Fig. 2) were obtained in the Laboratory of Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, through the courtesy of Dr. A. E. Kellie. The instrument used was Perkin Elmer double beam recording spectrometer (Model 21). The concentration of the free compound and also its acetate was approximately 1% in carbon disulfide solution. The brief analysis (Table I) of the infrared records indicates the groups responsible for some of the absorption bands; the assignments were done on an empirical basis (7).

DISCUSSION

The formula, $C_{30}H_{50}O_2$, of the compound isolated from petroleum ether extract of the drug, suggests that it may be a triterpenoid diol. A number of pentacyclic terpenes having the same formula, i. e., $C_{30}H_{50}O_2$, are known (8), but it is generally known that triterpenoid diols have high melting points while the compound under investigation has rather a low melting point, *viz.* 143–145°.

The compound contains hydroxyl grouping as seen from the infrared spectra and forms an acetate and a digitonide. The formation of a digitonide is generally considered to be a character of β-type configuration of hydroxyl group at C_3 in sterols (9), but triterpene alcohols also sometimes give digitonides (10). The isolated compound gives positive color tests of sterols (6).

The findings do not give a positive indication as to the classification of this compound as a sterol or a triterpenoid diol. Further work on the compound could not be done, due to its small amount. It has, however, been provisionally named "orthosiphonol."

SUMMARY

1. The whole plant of *Orthosiphon pallidus* Royle was subjected to proximate analysis and a series of extracts prepared.

2. Various qualitative tests were performed on the extracts of the drug:

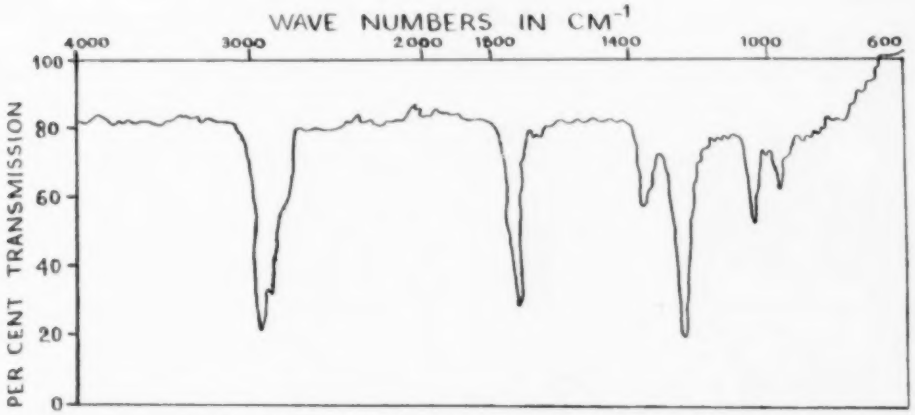

Fig. 2.—Infrared spectrum of the acetate of isolated substance.

TABLE I.—EMPIRICAL ASSIGNMENT OF GROUPS RESPONSIBLE FOR ABSORPTION BANDS IN THE INFRARED SPECTRA

Absorption Band Peak, cm.⁻¹	Assigned Grouping
	Fig. 1
3647	Hydroxyl (—OH)
2910, 2868	C — H
	Fig. 2
2930, 2865	C — H
1735	Carbonyl of Ester Group O ‖ R — C — OR′
1250	CH_3— of the acetate

(*a*) The alcohol and water extracts contained some alkaloidal matter, and (*b*) the presence of reducing sugars and disaccharides have also been indicated.

3. From the petroleum ether extract of the plant, a pure white crystalline substance has been isolated. It gives color reactions for sterols. The molecular formula of the compound has been found and derivatives prepared. The infrared spectra of the compound and its acetate, indicate that the isolated principle contains hydroxyl grouping. It is provisionally named "orthosiphonol."

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Investigation of *Orthosiphon pallidus* Royle II*

Isolation of Choline and Betaine

By N. K. BASU and HARKISHAN SINGH

The plant shows a variation in nitrogenous bases present. From the plant collected in April–May and September, choline has been isolated. Whereas the plant collected in July (rains) yielded betaine in addition to choline. The infrared spectra of the isolated bases are given.

IN THE PREVIOUS contribution (1) the plant was reported to contain some water-soluble bases. The isolation of a sterol-like compound and its preliminary physicochemical characterization have also been reported. Later, the plant was studied for its pharmacological activity. Its aqueous extract was shown to stimulate the isolated ileum and isolated virgin uterus of guinea pig, inhibit the pithed frog's heart and lower the blood pressure of the anesthetized dog. The action on isolated ileum of guinea pig and blood pressure of the anesthetized dog was found to be partially blocked by atropine.

Mithal, B. M. (2), isolated from the plant a water-soluble alkaloid (m. p. 112–113°), which due to its very minute quantity, could not be studied for its chemical characteristics or its pharmacological properties. The present experiments were undertaken in an attempt to isolate the previously reported alkaloid and also to search for the principles responsible for the pharmacological activity of the plant. Two bases were isolated and characterized. The presence of these bases has been found to vary in different seasons.

* Received February 27, 1956, from the Department of Pharmaceutics, Banaras Hindu University, Banaras, India.

EXPERIMENTAL

Isolation from First Batch.—The drug for this batch was collected locally in the months of April and May. Five kilograms of the drug (whole plant), in moderately coarse powder, was extracted with 90% alcohol by cold percolation. The solvent was recovered from the percolate and the concentrate obtained was treated with 3% sulfuric acid (1.8 liters). After being cooled in a refrigerator for a few hours the acid extract was filtered and treated with a saturated solution of ammonium reineckate. The precipitated alkaloidal reineckate was filtered, sucked dry (20.65 Gm.) and taken up with acetone (500 ml.). The acetone solution was treated with a 0.55% silver sulfate solution (800 ml.) followed with 80.0 ml. of 4.3% $BaCl_2 \cdot 2H_2O$ solution. The filtrate obtained after this operation was concentrated by distillation under vacuum, the syrupy liquid so obtained was dried in a vacuum desiccator and the residue was dissolved in absolute alcohol, filtered, and the filtrate dried again. In this way 6.8 Gm. of dirty brown "crude alkaloidal hydrochloride" was obtained.

The alcoholic solution of the crude alkaloidal hydrochloride was partially purified over a chromatographic column of alumina (100 Gm.) using 95% alcohol as solvent. The major bulk of the coloring matter (free from alkaloids) remained adsorbed on the top of the column and the alkaloidal portion flowed down as an orange brown liquid (400 ml.). No fractionation into different basic

entities occurred. Washing of the column with acidified alcohol indicated that the fraction adsorbed at the top was nonalkaloidal. The orange brown eluate, containing alkaloidal fraction, was treated with 15 Gm. of animal charcoal, kept overnight, and filtered. The animal charcoal adsorbed the coloring matter leaving a light yellow liquid, whereas it did not adsorb any alkaloids as seen by warming the alcohol-washed animal charcoal residue on the filter with diluted hydrochloric acid and testing the latter with Dragendorff's reagent. The light yellow alcoholic filtrate was evaporated to dryness at low temperature, the residue taken up with absolute alcohol, and filtered. On the addition of an excess of dry acetone to the filtrate a precipitate of colorless crystals formed. These crystals were further purified by repeated precipitation with acetone from absolute alcohol solution. The final dried crop weighed 0.92 Gm. (0.0184% of the air dried drug). It melted with decomposition between 288–290°. It gave positive alkaloidal tests with Dragendorff's reagent and ammonium reineckate solution. It was labeled as "Fraction A."

Second Batch.—The drug for the purpose was collected locally in the month of July (rainy season). Twenty kilograms of the powdered drug was worked up as given under "First Batch" up to the stage of getting the "crude alkaloidal hydrochloride." But here when the "crude alkaloidal hydrochloride" was taken up with absolute alcohol and filtered, there remained on the filter paper a yellowish-white organic nitrogenous residue which was sparingly soluble in absolute alcohol. This residue was kept separate and the filtrate after concentration was chromatographed on alumina, later treated with animal charcoal and eventually 0.49 Gm. (0.0025% of the air dried drug) of a substance having physical and chemical properties of the "Fraction A" was obtained, same practical details being followed as above.

The yellowish white residue, sparingly soluble in absolute alcohol, was crystallized from methyl alcohol and purified by recrystallizations from the same solvent. Finally a crop of colorless crystals, m. p. 227°, alkaloidal in nature, was obtained, weighing 0.913 Gm. (0.0046% of the air dried drug) and labeled as the "Fraction B."

Third Batch.—Ten kilograms of the drug, collected locally in the month of September, was extracted as in the "first batch" and 0.977 Gm. (0.0098% of the air dried drug) of the "Fraction A" was isolated. "Fraction B" was not found to be present in this batch of the drug.

Pharmacological Actions.—Before undertaking chemical characterization, the isolated hydrochlorides of the two bases were studied for their pharmacological action.

"Fraction A" was found to stimulate isolated ileum of guinea pig and isolated uterus of virgin guinea pig, inhibit the pithed frog's heart, and lower the blood pressure of the anesthetized dog. Action on isolated ileum of guinea pig and blood pressure of the anesthetized dog was examined after atropine and the latter caused complete blocking, showing that the isolated principle is muscarine-like in action.

The "Fraction B" inhibited the heart of pithed frogs; otherwise it was pharmacologically inactive. Figure 1 shows the response due to 0.1 ml. of 1:200 solution of the "Fraction B." There is a definite depression of the heart, amplitude of contraction is decreased, and the action is prolonged.

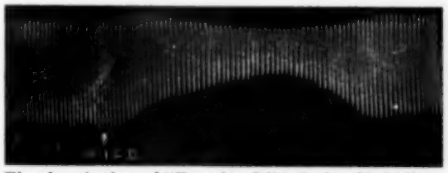

Fig. 1.—Action of "Fraction B" solution (1:200) on pithed frog's heart. Top indicates the base line.

Infrared Spectra.—The infrared spectra for "Fraction A" (Fig. 2) and "Fraction B" (Fig. 3) were taken in Nujol mull, using the instrument Baird Associates infrared recording spectrophotometer with sodium chloride prism. The spectra were obtained through the courtesy of Tapan Mukerjee of Wayne University (U. S. A.).

Chemical Characterization.—"*Fraction A.*"—*Anal.* Calcd. for $C_5H_{14}NOCl$: C, 43.01; H, 10.04; N, 10.04; Cl, 25.43. Found: C, 42.15; H, 10.01; N, 10.24; Cl, 25.11.

The base from this hydrochloride (Fraction A) was prepared by passing its solution in 60% alcohol over a regenerated column (20 Gm.) of E. Merck—Ion Exchanger III (3). The base so obtained was hygroscopic, like its hydrochloride, and gave the usual qualitative tests (4) for quaternary ammonium compounds. From these observations it appeared that the compound under study is choline chloride. Choline chloride (E. Merck) was found to melt between 285–294° with decomposition. About 10 mg. of choline chloride was mixed with about 10 mg. of the "Fraction A" with the aid of a little alcohol; the mixture was dried under vacuum. On determination of the melting point of this mixture it started changing color from 270° and melted with decomposition at 288–290°. One easily detectable difference between choline chloride and "Fraction A" was that the former had distinct disagreeable odor whereas the latter was odorless.

The reineckate derivatives both of "Fraction A" and "choline chloride" were prepared and were found to melt with decomposition at 250–252° and 251–252° respectively. The platinichloride derivatives were also prepared and found to melt at 235–236° and 237–238° respectively. It is stated that platinum salt of choline (5) has the composition $[(C_2H_4OH)(CH_3)_3NCl]_2, PtCl_4$, in which Pt constitutes 31.68% of the whole molecular weight, and it was experimentally found that the platinichloride of "Fraction A" leaves on ignition 31.05% of the residue (Pt).

One-eighth gram of the "Fraction A" was mixed with 2.5 Gm. of soda lime and the mixture was heated in a Pyrex bulb over Wood's metal bath, under a current of nitrogen. The evolved fumes were entrapped in alcoholic HCl solution and from the residue of the latter a picrate (m. p. 214–216°) was prepared. The melting point is quite close to the melting point of trimethylamine picrate (m. p. 216°) (6).

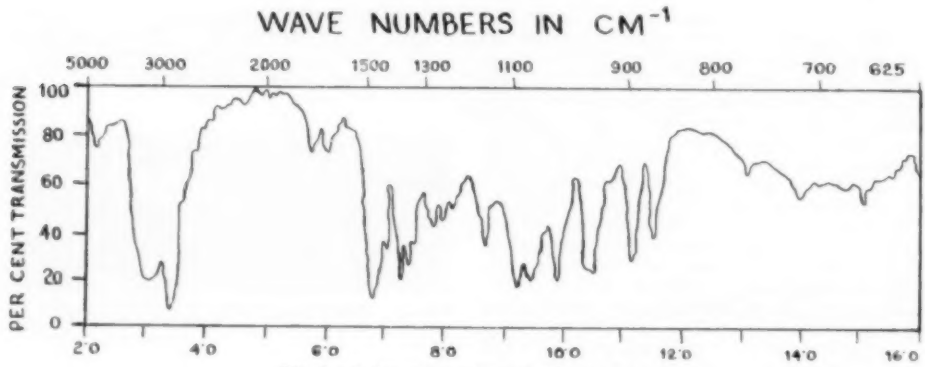

Fig. 2.—Infrared spectrum of "Fraction A."

"Fraction B."—Anal. Calcd. for $C_5H_{11}NO_2 \cdot HCl$: C, 39.09; H, 7.82; N, 9.12; Cl, 23.10. Found: C, 39.89; H, 7.67; N, 9.19; Cl, 22.80.

The base was prepared by decomposing the hydrochloride (Fraction B) by rubbing it with freshly prepared moist silver oxide, the liberated base being taken up with methyl alcohol. The base was purified by repeated crystallization from absolute alcohol. A crop of colorless crystals was obtained (m. p. 264–265° decompn.).

Anal. Calcd. for $C_5H_{11}NO_2$: C, 51.29; H, 9.40; N, 11.96. Found: C, 52.05; H, 9.25; N, 12.13. Molecular weight found (by cryoscopic method with water as solvent); 118.2. Calcd. for $C_5H_{11}NO_2$: 117.

The derivatives, reineckate (m. p. 148–150°) decompn.) and picrate (m. p. 181–182°), were prepared.

On reference to literature (7, 8) it was found that hydrochloride of betaine has a similar formula, *viz*, $C_5H_{11}NO_2 \cdot HCl$ and melts (7) at 228°. The base, betaine melts (8) with decomposition at 273° ± 3° and its picrate (7, 8) at 180–181°. From this data it appeared that the "Fraction B" is betaine hydrochloride. Mixed m. p. with betaine hydrochloride (B. D. H.) was found to be 227–228°. The reineckate derivative of betaine hydrochloride (B. D. H.) was prepared and found to melt with decomposition between 149–150°.

The "Fraction B" (0.272 Gm.) was treated with soda lime (2.5 Gm.) as per details given under "Fraction A" and picrate (m. p. 216°) of the decomposition product obtained. The mixed melting point of this picrate and that obtained under "Fraction A" was undepressed, proving both to be the same entity.

DISCUSSION

The two isolated compounds, *viz.* "Fraction A" and "Fraction B," have been characterized as choline chloride and betaine hydrochloride respectively. In addition to their chemical behavior, the infrared spectra of both are in accord with the above conclusion, though a comparative study of these vis-à-vis standard spectra of choline chloride and betaine hydrochloride could not be made, as they were not available.

Choline, is widely distributed (9, 10) and it is known to have qualitatively the same action as acetylcholine but is much less active (11). The results of the pharmacological study of choline chloride isolated from *Orthosiphon pallidus* Royle are in agreement with the known pharmacological actions of choline.

Betaine, trimethylglycolcoll, is also common in

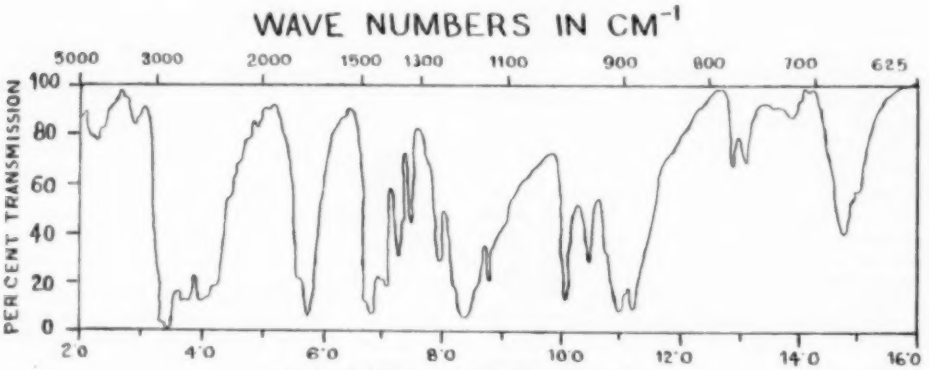

Fig. 3.—Infrared spectrum of "Fraction B."

nature (12, 13). Waller and Plimmer find that betaine is feebly but distinctly toxic. In a dose of 0.1 Gm./Kg. it produced in rabbits fall of blood pressure with final paralysis of the heart (14). But no lowering of blood pressure was noticed in anesthetized dogs, on intravenous administration of betaine hydrochloride (Fraction B) isolated from *Orthosiphon pallidus* Royle. This, in all probability was due to a very small dose given (2 ml. of 1:500 solution to a dog weighing 6.1 Kg.). However it produced definite inhibition of heart of pithed frog.

Choline and betaine are very closely related to each other chemically (15). It has been mentioned in the present communication, that the plant (*Orthosiphon pallidus* Royle) contains only choline in some seasons (April-May and September), while in the rainy season (July) it contains both choline and betaine. Possibly some biochemical change occurs in the plant during the rainy season leading to the oxidation of choline to betaine, which after rains is again transformed into choline or mobilized in some other way with the result that the September sample of the drug again yields only choline.

The alkaloid isolated by Mithal (2) seems to be different from choline and betaine. The present authors have not found it in the plant. It is very probable that the alkaloid isolated previously occurs in this plant during seasons other than those in which it was collected for the present work.

The problem of searching for the alkaloid isolated previously and other pharmacologically-active constituents still remains an open question. Work on this plant continues to be in progress in this laboratory.

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A Radioactive Tracer Technique for Determining the Release of Medication from Hydrophilic Ointment Bases*

By DONALD Y. BARKER, JOHN E. CHRISTIAN, and H. GEORGE DeKAY

This paper describes the technique, details of experimentation, and the results obtained when several hydrophilic ointment bases of current interest were tested by a modified agar-plate method using radioactive tracer techniques.

WHEN THE RELEASE of medication from ointment bases is determined by the agar-plate method, the bacteriostatic effect of the incorporated medicinal agent on a standard culture organism is employed as the criterion for evaluation of the results. This method, however, gives no indication of the actual amount of medication that diffuses from the ointment bases nor does it indicate whether the medicament diffuses beyond the indicated zone of inhibition.

A procedure employing radioactive tracer techniques would possess definite obvious advantages. Accordingly, this method was developed using basically the same fundamental processes and techniques as are involved in the agar-plate method. Instead of using the bacteriostatic efficiency of the medicament as the criterion for

* Received April 5, 1956, from the Bionucleonics Laboratory, School of Pharmacy, Purdue University, Lafayette, Indiana.
Presented to the Pharmacy Section, A. A. A. S., Atlanta Meeting, December, 1955.

release of medication, a known amount of labeled medicament was incorporated into the ointment and the total release and distribution of the medicament was determined by radioactivity measurements.

EXPERIMENTAL

Choice of Radioactive Isotope.—Iodine-131 was selected as the radioisotope primarily because iodine was employed as one of the medicaments when the effect of nonionic surfactants on the release of medication from hydrophilic ointment bases was determined (1).

In addition, iodine-131 has a convenient half-life of eight days and is easily detected and measured.

Standardization of the Sodium Radio-Iodide Solution.—The iodine-131 was available as sodium radio-iodide[1] in solution with sodium bisulfite.

The procedure employed for the standardization of the sodium radio-iodide solution and the preparation and standardization of the ointments was basically a modification of the method employed by Skauen, *et al.* (2), and Cyr, *et al.* (3). The equipment used for counting all of the samples was as follows: A Scaler, Model 165[2] was attached to an Automatic Sample Changer, Model C-110[2] and a Printing Timer, Model C-111,[2] which recorded the time for a preset number of counts. The activity of each sample was corrected and expressed as counts per minute.[3] One milliliter of the stock solution, used in subsequent experiments was found to contain 5,000,000 CPM per ml.

Choice of Nonionic Surfactants Used for Testing the Hydrophilic Ointment Bases.—From the results of the agar-plate study (1), three surfactants exhibiting a superior ability to release medication from the ointment bases tested were selected for the preparation of the hydrophilic ointment bases employed in this study. These surfactants were G-7596-J[4], Brij[5] 35,[4] and Ethomid C/15[5].

Hydrophilic ointment bases were prepared from these nonionic surfactants in three concentrations 1, 3, and 5%, employing the same formulation as used in the agar-plate study.

Preparation of the Ointments. The formulation for the ointments to be tested was as follows:

Sodium radio-iodide	12,500,000 CPM[6]
Iodine	0.05 Gm.
Potassium iodide	0.05 Gm.
Ointment base	4.90 Gm.

The sodium radio-iodide solution contained on a watch glass was dried under infrared light. To the dried residue was added approximately 0.1 ml. of dilute sulfuric acid to oxidize the iodide ion to molecular iodine. This solution was allowed to stand for one to two hours and the finely powdered potassium iodide dissolved in it. The iodine, previously pulverized, was then dissolved in this potassium iodide solution and the solution incorporated into a portion of the weighed amount of the ointment base, care being taken to remove the last traces of activity from the watch glass with repeated applications of the remainder of the ointment base. The ointment was then dispensed in a one-dram collapsible ointment tube for ease in application to the agar cup.

Standardization of the Ointment—Each sample of ointment was standardized to determine the amount of activity in a definite weight of ointment. An accurately weighed sample approximating 0.15 Gm. was transferred to a 100-ml. volumetric flask. About 20 ml. of warm distilled water was added to disperse the active ingredient and the isotope throughout the solution. When cool, the volume was adjusted to 100 ml. with 95% alcohol. Six, 100-lambda samples of this solution were added to aluminum counting cups lined with a layer of Whatman filter paper. The samples were air dried, after which they were counted to a preset number of counts. By multiplying the mean corrected CPM by the dilution factor, the activity of the accurately weighed sample of ointment was determined.

Testing the Ointments.—The agar plates were prepared in the usual manner except the nutrient agar was not inoculated with the standard culture and only one cup was placed in the center of the agar plate.

Each sample of ointment dispensed to the agar cup was weighed by a method of differences in the weight of the tube containing the ointment before and after the sample of ointment had been dispensed. Four samples of each ointment were subjected to testing. The petri dishes containing the agar and the ointment samples were inverted and incubated for a period of twenty-four hours at 37°. After the incubation period, the agar was removed from the petri dishes, intact, and placed on a piece of waxed paper.

A strip of agar, 10 mm. in width which passed through the ointment sample was cut. Perpendicular to this strip of agar and intersecting at the ointment sample, another strip of agar, 10 mm. in width was cut. The four quadrants of agar adjacent to the agar strips were removed and discarded leaving, after the central area of agar containing the ointment sample had been removed, four strips of agar, 40 mm. by 10 mm. Each strip of agar was cut at 5-mm. intervals along the 40-mm. length producing agar samples 10 mm. by 5 mm. Each sample of agar was placed on a previously prepared counting cup, allowed to air dry and then counted to a preset number of counts. The CPM were calculated and then corrected for background and decay using the correction factors of Pinajian and Christian (4). No resolving time corrections were made as the samples were not sufficiently active to warrant such corrections.

For each ointment sample tested, the corrected CPM for the four agar samples at each 5-mm. distance interval from the ointment sample was totaled. This represented the activity in each 200 sq. mm. of agar at eight progressive 5-mm. distance intervals from the ointment sample.

To calculate the activity in the total area of each

[1] Received on allocation from Atomic Energy Commission, Oak Ridge, Tenn.
[2] Available from Nuclear Instrument and Chemical Corp., Chicago, Ill.
[3] Counts per minute, hereinafter referred to as CPM.
[4] Trademark name of Atlas Powder Co., Wilmington, Del.
[5] Trademark name of Armour and Co., Chicago, Ill.
[6] This activity of the isotope was selected because an activity of 500,000 CPM per 0.2 Gm. sample of ointment was deemed sufficient for this study. The volume of the stock solution to produce such an activity was 2.5 ml.

5-mm. wide concentric circle of agar, the activity in each 200 sq. mm. of agar was multiplied by a calculated factor. These respective factors were derived by dividing the total areas in each consecutive concentric circle of agar by 200.

The per cent medication in each 5-mm. wide concentric circle of agar for the four samples of each ointment base tested was averaged, thus indicating the distribution of the medication in the agar. The mean per cent of medication in the eight concentric circles of agar was then totaled representing the total release of medication from the ointment bases into the agar medium. The results are indicated in Table I.

The radioactive tracer technique for testing diffusion of medication from ointment bases possesses definite advantages over the conventional agar-plate method. The major advantage is the direct determination of the actual amount and the distribution of the medication released to the agar from the ointment bases. A phenomenon that is not indicated by the agar-plate method is the fact that the medicament diffused throughout the agar medium. The zone of inhibition gives an indication of the area in the agar medium in which the concentration of the medicament is sufficient to exert its

TABLE I.—MEAN PER CENT OF MEDICAMENT PRESENT IN EACH 5-MM. WIDE CONCENTRIC CIRCLE OF AGAR AFTER TWENTY-FOUR HOUR'S EXPOSURE TO APPROXIMATELY 20 MG. OF HYDROPHILIC OINTMENT CONTAINING ONE PER CENT OF I-131 LABELED IODINE AND PREPARED WITH VARIOUS SURFACTANTS

Surfactant and Concentration, %	Total Medication in Indicated Area of Agar %,[a] Distance Interval from Ointment, mm.[b] 0–5	5–10	10–15	15–20	20–25	25–30	30–35	35–40	Total Medication Released to Agar, %[c]
G-7596-J 1	9.46	12.05	13.32	12.61	10.83	9.12	7.19	6.64	81.2
3	9.34	10.20	11.00	10.72	9.19	8.14	6.23	5.55	70.4
5	13.69	13.90	13.58	12.40	10.86	8.07	6.67	5.04	84.2
Ethomid C/15 1	9.29	10.96	12.58	12.39	11.98	10.23	9.27	8.62	85.3
3	10.26	11.51	11.19	10.93	9.26	7.54	5.47	5.24	71.4
5	10.76	12.00	12.15	11.81	10.54	7.93	6.38	4.97	76.5
Brij 35 1	11.43	13.42	13.36	12.61	10.48	8.54	6.89	5.78	82.5
3	6.23	6.43	6.79	6.27	5.31	4.46	3.62	2.76	41.9
5	7.27	6.87	6.68	5.73	4.92	3.29	2.78	2.20	39.7
Hydrophilic ointment U. S. P. XIV	9.60	12.14	13.45	13.33	12.44	9.12	8.47	7.33	85.9

[a] This value represents the mean of the calculated per cent of total incorporated medicament contained in each 5-mm. wide concentric circle of agar when four samples of each ointment base were tested.

[b] The distance indicates the 5-mm. wide concentric circles of agar at indicated distance intervals from the sample of ointment.

[c] This value indicates the total of the mean value of the per cent of medicament contained in each 5-mm. wide concentric circle of agar for the four samples of ointment tested.

DISCUSSION

In this instance, when the per cent release of medication from each ointment base in the three concentrations was totaled, the surfactant G-7596-J followed by Ethomid C/15 and Brij 35 exhibited maximum release of incorporated medication. Since the surfactant and its concentration are the only variables in the formulation and procedure for testing the release of medication, the difference in this release of medicament can be attributed to the surfactant and its concentration in the ointment base formulation. This trend in the results obtained by the modified method was the same as when the ointment bases were tested by the agar-plate method (1) except when hydrophilic ointment U. S. P. XIV was tested.

Maximum release of medication was obtained when the surfactants were employed in a 1% concentration in the ointment base formulation. The only exception was the surfactant G-7596-J which gave maximum release of medication when employed in the ointment base formulation in a 5% concentration. However, this identical trend was indicated when the ointments were tested by the agar-plate method.

The zone of inhibition produced in the agar-plate method, therefore, does not indicate the true picture of medicament release.

This fact was especially noted when the standard ointment, hydrophilic ointment U. S. P. XIV was tested. The zones of inhibition produced by this ointment base were smaller than those produced by the other ointment bases tested when the surfactant was employed in a 1% concentration. This ointment, however, when tested by the radioactive tracer technique, was shown to release incorporated medication to a greater extent than all other bases.

It is postulated that because sodium lauryl sulfate, the surfactant in the formulation, possesses increased surface tension-reducing properties over the nonionic surfactants, more water is available in the ointment base for release of incorporated medication. This increased availability of water probably accounts for the increased ability of the ointment to release the medicament.

The smaller zone of inhibition produced in the agar plate may possibly be explained by the fact that medicament release is more rapid and distribution throughout the agar occurs more completely, lowering the concentration below that necessary to exert bacteriostasis except within the noted zone of inhibition.

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Pharmacological Effects from Drugs Injected Intracerebrally in Unanesthetized Animals*

By THOMAS J. HALEY

The intracerebral injection of cardiac glycosides, quinidine sulfate, procainamide, chlorpromazine, reserpine, and lysergic acid diethylamide in unanesthetized dogs produced symptoms of a profound autonomic nervous system stimulation. Many common effects were produced by several structurally unrelated drugs. However, certain drugs had rather specific actions, which seemed to be localized in discrete areas in the brain. Studies with such preparations allow an estimate to be made of central activity without the complicating factors of general anesthesia or diffusion across the blood brain-barrier.

MANY DRUGS have diverse effects on the central and autonomic nervous systems, and by the large it is difficult to separate their central from their peripheral effects. In many instances diffusion of drugs into the central nervous system is so slow that central effects appear to be entirely absent or appear only at toxic doses. Furthermore, studies are often conducted in the presence of general anesthesia which tends to partially or completely obscure any central effects. In the past many investigations have been conducted on curarized, artificially respired animals in an effort to obtain estimates of central activity of drugs. Recently, Feldberg and Sherwood (1–4) devised a cannula for injection of drugs into the lateral ventricle of the conscious cat. Haley and co-workers (5–9) employed a similar cannula in studies with the unanesthetized dog. It is the purpose of this paper to summarize these dog experiments to date and point out the similarities of response elicited by drugs of unrelated chemical structure as well as the differences in response which appear to be related to certain drugs. In addition an attempt will be made to relate pharmacological effects with possible sites of action in the brain.

EXPERIMENTAL

Healthy, adult dogs of either sex were prepared for implantation of a cerebral cannula according to the method described previously (3, 9). Aspiration of cerebrospinal fluid as well as roentgenograms showed that the cannula penetrated into the third cerebral ventricle. Intraventricular injections varied in volume from 0.2 to 1 ml. Intravenous injections within comparable dosage ranges were made via the anterior subcutaneous vein of the foreleg. Electrocardiographic records were obained with lead II using a Sanborn Viso-Cardiette. Figure 1 shows a schematic drawing of the needle in position and some of the areas in the ventricular system which could be affected by the injected drugs.

RESULTS

Cardiac Glycosides.—Intraventricular injection of 3.2 to 4.4 mcg./Kg. of tryptamine-strophanthidin or 6.4 to 8.4 mcg./Kg. of strophanthin-K produced bradycardia and depression of the ST segment of the electrocardiogram characteristic of digitalis. Secondary effects were: salivation, defecation, mydriasis, shivering, generalized tremor, and mucous membrane and retinal pallor. All of these effects disappeared after 11 minutes and the animals appeared normal. With larger doses, 10.9 to 17.2

* Received February 27, 1956, from the Division of Pharmacology and Toxicology, Atomic Energy Project and the Department of Medicine, School of Medicine, University of California, Los Angeles.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April, 1956.
This research was supported in part by Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles, and in part by a grant from Ciba Pharmaceutical Products, Inc.
The author wishes to thank Dr. H. W. Magoun and Mr. W. G. McCormick for their assistance; Sandoz Pharmaceuticals for the LSD-25 and the Strophanthin-K; Smith, Kline and French Laboratories for the chlorpromazine; and Ciba Pharmaceutical Products, Inc., for the reserpine used in this investigation.

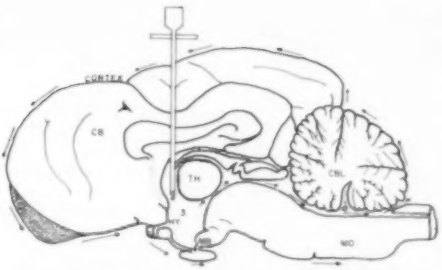

Fig. 1.—Median sagittal view of dog brain showing indwelling cannula. Arrows indicate circulation of cerebrospinal fluid; cerebrum, CB; cerebellum, CBL; thalamus, TH; hypothalamus, HY; third ventricle, 3; fourth ventricle, 4; mammillary body, MB; medulla, MO.

mcg./Kg. of tryptamine-strophanthidin or 31.4 to 57.4 mcg./Kg. of strophanthin-K, ventricular extrasystoles and paroxysmal ventricular tachycardia appeared in three minutes. After two to nine minutes, some animals had bigeminy, trigeminy, sinus tachycardia, multifocal ventricular tachycardia and periodic P-wave suppression. Death resulted from respiratory paralysis and ventricular fibrillation. Additional secondary effects were: barking, generalized excitement, lateral and vertical nystagmus, opisthotonus, tense abdominal musculature, retching, emesis, micturition, tachypnea, and orbicularis muscle twitching. These effects must be centrally induced because intravenous injection of 8.9 to 9.8 mcg./Kg. of tryptamine-strophanthidin or 31 mcg./-Kg. of strophanthin-K had no effect. Doses of 20.1 to 26.8 mcg./Kg. of tryptamine-strophanthidin produced a deep Q-wave and a biphasic T-wave or an inverted T-wave with a late sagging ST segment and a bradycardia. Such effects had a duration of only ten minutes. Strophanthin-K, 77 mcg./Kg. produced only a bradycardia of nine minutes duration. Secondary effects were not observed.

Injection of the vehicles into the third ventricle produced no effect. The intravenous administration of 15 to 37.3 mg./Kg. of hexamethonium chloride completely abolished the secondary effects and re-established normal cardiac rhythm within eight minutes. Chlorpromazine, 20 mg./Kg., also was effective. Furthermore, anesthetization with pentobarbital decreased the cardiac rate and induced a regular sinus rhythm.

Quinidine Sulfate.—Intraventricular injection of quinidine sulfate in doses ranging from 0.45 to 4.0 mg./Kg. produced paroxysmal ventricular tachycardia, alternating nodal extrasystoles or auricular tachycardia followed by a diminishing sinus tachycardia. Immediate secondary effects consisted of retching, micturation, defecation, mydriasis, nystagmus, respiratory depression, mucous membrane pallor and Stage III, Plane II general anesthesia with corneal reflexes still present. None of these effects were seen after intravenous injection of 2.8 to 8.1 mg./Kg. of the drug. Furthermore, intraventricular injection of 0.5 ml. of propylene glycol, which composed 20% of the vehicle, produced none of the above effects. Quinidine sulfate did not cause any fatalities.

Procainamide.—Intraventricular injection of 5.2 to 7.1 mg./Kg. of procainamide had little or no effect on the electrocardiogram. Side effects were limited to defecation, nystagmus, slight twitching of the facial musculature and Stage III, Plane II anesthesia. None of these effects were seen after 10.3 mg./Kg. intravenously.

Chlorpromazine.—Intraventricular injection of 0.3 mg./Kg. tranquilized the animal for 16 minutes and produced a lowered, biphasic T-wave. No other effects were observed. When the dose was increased to 0.93 to 1.10 mg./Kg. the following cardiac effects occurred: bradycardia; an inverted, lowered or biphasic T-wave; followed by tachycardia; series of one, two, or three ventricular extrasystoles and coupled normal beats. After about 20 minutes the T-wave became upright. Side effects were: analgesia and tranquilization, shivering, decreased respiratory rate, defecation, mydriasis and occasional barking. Body temperature fell 0.3 to 1.6° over a period of 35 minutes and then returned to normal 15 minutes later. Ten to 12 hours later the animals appeared normal. Intravenous injections of 20 mg./Kg. produced variability in the height of the T-wave with tachycardia followed by bradycardia. Normal electrocardiograms were obtained 11 to 20 minutes later. Secondary effects were: attempted emesis, defecation, vertical and lateral nystagmus, miosis with reactive pupils, ataxia, micturition, and analgesia with no reaction to painful stimuli. Body temperature decreased 0.6° and a slight respiratory depression occurred.

Reserpine.—Intraventricular injection of 2.2 to 4.5 mcg./Kg. produced salivation, retching, and emesis. No other effects were observed, and the animals appeared normal 20 minutes after the injection. When the dose was 25 mcg./Kg. the following effects were observed: salivation, repeated emesis, slight miosis, and defecation. Electrocardiographic changes were tachycardia, biphasic or inverted T-wave, and an accentuated P-wave. The animals were normal within 24 hours.

Lysergic Acid Diethylamine (LSD–25).—Intraventricular injection of 1.6 to 10 mcg./Kg. produced the following symptoms within one to three minutes: whining, shaking of the head, salivation, retching, emesis, micturition, tachypnea, and ataxia. After six minutes, mydriasis with reactive pupils was observed. Recovery occurred after 15 to 20 minutes, but throughout this period the animals appeared frightened. The animals barked for several hours after the other effects of the drug were gone. The animals reverted from an adult behavior to a puppy one, but there was no impairment of their ability to obey commands or perform simple tasks. This personality change was the most striking effect which occurred.

DISCUSSION

The various pharmacological effects produced by intracerebral injection of drugs in the unanesthetized dog are summarized in Tables I and II. The possible sites of action listed should not be considered as the only sites because activation of receptors in the cerebral ventricles may, through interneuronal pathways, cause more distant centers to be affected. These centers may give rise to the observed effects which are for the most part evidence of a profound

TABLE I.—COMMON EFFECTS FROM INTRACEREBRAL INJECTION OF DRUGS

Effect	Strophanthin-K and Tryptamine Strophanthidin	Procainamide	Quinidine Sulfate	Chlorpromazine	Reserpine	LSD-25	Possible Sites of Action
Salivation	+	+	−	−	+	+	Pre-optic hypothalamus
Retching	+	−	+	−	+	+	Brain stem reticular formation
Emesis	+	−	−	−	+	+	Chemoreceptor trigger zone
Mydriasis	+	−	+	+	−	+	Posterior hypothalamus
Nystagmus	+	+	+	−	−	−	Pontine area or medial longitudinal fasciculus
Defecation	+	+	+	+	+	−	Hypothalamic, pre-optic and supra-optic nuclear areas
Micturition	+	−	+	−	−	+	Pre-optic hypothalamus
Vasoconstriction	+	−	+	−	−	−	Posterior hypothalamus, vasomotor center in medulla
Shivering	+	−	−	+	−	−	Posterior hypothalamus
Tachypnea	+	−	−	−	−	+	Respiratory center in medulla
Bradypnea	−	−	−	+	+	−	Respiratory center in medulla

+ Present; − absent.

autonomic nervous system activation. Furthermore, it would be inconsistent to insist that certain specific sites in the walls of the third ventricle are the only ones being affected because the brain ventricles form a closed system and the drugs eventually will spread throughout the entire system. The possibility of diffusion into the mass of brain tissue surrounding the ventricles also could play a part in the overall effects observed. As can be seen in Table I, there are many drug effects produced which cannot be related to the chemical structure of the compounds studied. It may be that certain areas in the brain are more susceptible to the drugs than others. On the other hand certain drugs appear to have very specific effects on certain areas (see Table II). Correlation between the central sites of action and the observed peripheral effects may be obtained by comparing our results with those in which the electrophysiological approach has been used.

brain stem reticular formation. Borison and Wang (12) have pointed out the distinction between retching and emesis, relating the latter effect to an activation of the chemoreceptor trigger zone in the wall of the fourth ventricle. From this zone interneuronal fibers activate the emetic center in the dorsal portion of the lateral reticular formation, producing emesis. This may not be the only area involved because Hess (13) has shown that stimulation of the caudal portion of the diencephalon also produces retching and emesis. Ependymal receptors in the lateral ventricles also cause emesis when stimulated (12).

Hess (14) produced mydriasis by stimulation of the posterior hypothalamus, but centers for this activity also exist in the cerebral cortex and the brain stem. In all probability intracerebrally injected drugs act directly on the hypothalamic centers and then the impulses are conducted to the other centers of mydriatic activity. Miosis occurs after

TABLE II.—UNCOMMON EFFECTS FROM INTRACEREBRAL INJECTION OF DRUGS

Drug	Effect	Possible Site of Action
Strophanthin-K and tryptamine-strophanthidin	Abnormal electrocardiogram ending in ventricular fibrillation. Generalized excitation	Anterior and posterior hypothalamus
Procainamide	Stage III, Plane II general anesthesia with corneal reflexes present	Ascending reticular system; hypothalamus
Quinidine sulfate	Stage III, Plane II general anesthesia with corneal reflexes present	Ascending reticular system; hypothalamus
Chlorpromazine	Analgesia and tranquilization. Hypothermia, bradycardia followed by tachycardia	Hypothalamus and brain stem, thermoregulator center, posterior and anterior hypothalamus
Reserpine	Miosis	Optic chiasma, optic tract, pretectal region
LSD-25	Ataxia. Change from adult to puppy behavior pattern	Unknown

Furstenberg and Crosby (10) have related salivation to stimulation of the pre-optic hypothalamus. However, the salivatory nuclei in the medulla may also be stimulated. This may be of particular importance because of the well known relationship between salivation and emesis. Magoun (11) has correlated the postural changes accompanying retching with a facilitatory and inhibitory system in the

stimulation of the optic chiasma, the optic tract or the superior quadrigeminal brachium. Ranson and Magoun (15) also obtained this effect after stimulation of the pretectal region, the posterior commissure or the fibers emerging from the posterior commissure that arch around the ventral aspect of the central gray matter at the upper end of the cerebral aqueduct. Intracerebrally injected

drugs could reach and directly or indirectly activate any or all of these areas. Nystagmus, generally considered to be a pontine effect, may have been brought about through a collateral effect on the medial longitudinal fasciculus, which is represented in the hypothalamus by a nucleus in the posterior commissural area.

Defecation may be considered a peripherally mediated action, but Kuntz (16) states that experimental evidence suggests the existence of a defecation center in the medulla oblongata and of a center in the mesencephalon which exerts a tonic influence on the rectal musculature. Sheenan (17) has summarized the available evidence relating gastrointestinal hyperfunction to stimulation of the hypothalamic preoptic and supra-optic nuclear areas. Uvnas (18) reported that stimulation of certain areas in the hypothalamus results in micturition. However, tonus of the bladder musculature is also regulated through centers in the rostral border of the hindbrain and these centers are subject to influences from the cortical areas of both cerebral hemispheres. Autonomic centers in the diencephalon also influence bladder function.

Mucocutaneous vasoconstriction may be brought about by stimulation of the sympathetic areas of the posterior hypothalamus and also through activation of the vasoconstrictor center in the medulla. Blair and Keller (19) have related shivering to the posterior hypothalamus with its descending pathway through the lateral hypothalamus passing dorsolaterally to the mammillary body and entering the mesencephalic tegmentum. Stimulation of hypothalamic nuclei in the caudal border of the lateral hypothalamus results in hypothermia. The changes in respiratory rate observed may have been related to effects on the respiratory center in the medulla. The changes seen in the electrocardiographic tracing and heart rate are probably due to stimulation of the hypothalamus. Hess (14) has shown that a cardioinhibitory center exists in the anterior hypothalamus and a cardioacceleratory center exists in the posterior hypothalamus.

States of consciousness have been summarized by Magoun (20) as being related to the ascending reticular activating system adjacent to and having effective collaterals in the hypothalamus. Activation of this system could explain the general anesthesia and perhaps the analgesia and tranquilization observed.

As can be seen from the preceding discussion the areas of the brain which can be affected by intracerebrally injected drugs are numerous and diverse in activity. The results obtained by such injections are of central origin because the rapidity of onset of action and the amount of drug producing the observed effects are such to rule out peripheral sites of action. Much larger parenteral doses are required to produce effects and in many instances the effects are diametrically opposite to those obtained by intracerebral injection. Furthermore, the blood-brain barrier would effectively prevent the intracerebrally injected material from entering the blood stream rapidly thus further reducing the overall concentration which could affect the peripheral receptors. The vehicles alone did not produce the effects observed so they must be related to the drugs themselves.

SUMMARY

(1) Studies have been made of the effects produced by the injection of drugs into the third cerebral ventricle of unanesthetized dogs.

(2) Cardiac glycosides, quinidine sulfate, procainamide, chlorpromazine, reserpine, and lysergic acid diethylamide all produced certain effects in common but each drug also appeared to be able to affect certain specific areas in the brain.

(3) The possible sites of action in the brain were discussed and the difficulties of exact localization of such sites of action pointed out.

(4) The intraventricular injection of drugs in the unanesthetized animal allows a true assessment of central activity at doses which produce little or no effect when administered intravenously. Furthermore, the effects produced are not related to the vehicles used.

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Study of Stability of Sulfadiazine Sodium Injection I.*

Determination of Solubility of Sulfadiazine Sodium as a Function of *p*H in Several Parenteral Solvent Systems

By F. S. HOM and J. AUTIAN†

An investigation was conducted to stabilize sulfadiazine sodium injection when exposed to various storage conditions. Prolonged storage of sulfadiazine sodium injection U. S. P. has been known to result in coloration due to oxidation and formation of a precipitate. This study deals with the relationship of sulfadiazine sodium solubility with the hydrogen ion concentration in several solvent systems which have been employed in injectable products. Solubility curves were determined for a number of solvents and from these results their usefulness was ascertained.

THE ARMED SERVICES Medical Procurement Agency has received a number of complaints concerning the stability of sulfadiazine sodium injection U. S. P. from Service Hospitals throughout the United States. The two chief complaints were that a coloration and a precipitation formed on long standing.

Searching the literature disclosed little information regarding the stability of sulfadiazine solutions or for that matter any sulfa drug solutions. A great many texts and volumes of articles have been published dealing with the clinical and pharmacological actions of this drug; however, very little evidence has been gathered on the stability problem. Whittet (1) studied a number of sulfonamide injections and reported that all common sulfonamide injections were very sensitive to light and darkened in presence of light.

It is quite possible that the coloration of sulfadiazine is due to a decomposition product produced by a base catalyzed oxidation in the presence of sunlight. Seikel(2) and Barkan (3) have reported oxidation degradation products of sulfanilamide. Pigments were isolated by James (4) when sulfanilamide was oxidized with hydrogen peroxide.

A precipitate in sulfadiazine sodium injection may result from a change in the hydrogen ion concentration of the solution. Since the solubility of sodium sulfadiazine is 1 Gm. in 2 ml. of water and the solubility of the acid is 1 Gm. in 13,000 ml. of water, it may be inferred that a slight reduction in *p*H of the solution would throw out of solution some of the salt in the acid form as a precipitate.

The first portion of the present study deals with the investigation of the relationship of sulfadiazine sodium solubility with the hydrogen ion concentration in a number of solvents which have been utilized in injectable products. The object of this phase of the problem was to determine if a vehicle could be found which had a good solvent action on the drug at lower hydrogen ion concentrations. It is assumed that lower *p*H values would retard decomposition of the drug.

EXPERIMENTAL

Supplies and Apparatus.—Sulfadiazine sodium U. S. P., alcohol U. S. P., glycerin U. S. P., propylene glycol U. S. P., sorbitol (Atlas Powder Co., Wilmington, Del.), polyethylene glycol 200 and 400 (Carbide & Carbon Chem. Co., New York, N. Y.).

Beckman *p*H Meter, Model G, constant temperature water bath, vacuum filtration apparatus with a

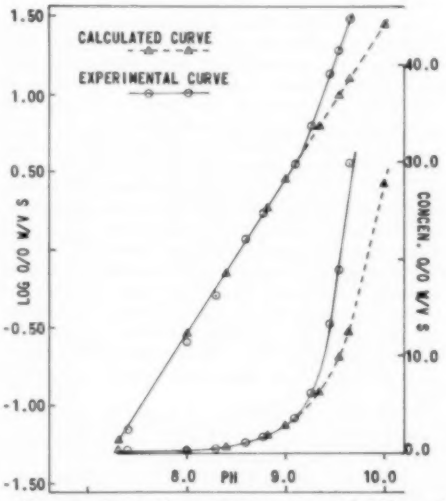

Fig. 1.—Two plots showing the solubility of sodium sulfadiazine in water at 25° C. as the function of *p*H.

* Received April 17, 1956, from the Temple University School of Pharmacy, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 1956.
This research project was conducted under contract from the Armed Services Medical Procurement Agency.
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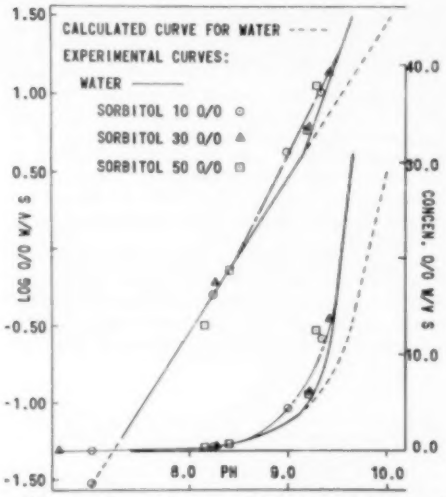

Fig. 2.—Two plots showing the solubility of sodium sulfadiazine in water and in sorbitol-water systems at 25° C. as the function of *p*H.

fused-in Gooch crucible, fritted disk filter of medium porosity.

Solubility of Sulfadiazine Sodium in Water as Function of *p*H.—A theoretical solubility curve was plotted by the use of the buffer equation:

$$pH = pKa + \log \frac{\text{salt}}{\text{acid}}$$

From this curve at a point 0.2 *p*H unit higher than the *p*H to be used, the amount of sulfadiazine sodium was determined. The sulfadiazine sodium was weighed on a prescription balance and placed in a 100-ml. beaker. Enough glass-distilled water was added to bring the volume of solution to measure 45 ml. One normal HCl was then added dropwise to obtain the desired *p*H. At all *p*H points, an excess of the salt was maintained. The final volume of solution was adjusted to 50 ml. in a cylindrical graduate. The solution was placed in a multiple-dose vial and stoppered with a rubber closure. After placing the vials in a constant temperature water bath maintained at 25° ± 0.2° they were agitated intermittently for forty-eight hours. Each vial was opened and its contents filtered through a medium porosity sintered glass filter at a reduced pressure. Approximately 10 ml. of the filtrate was then set aside for assay by the official method (4). A *p*H determination was also recorded for the filtrate.

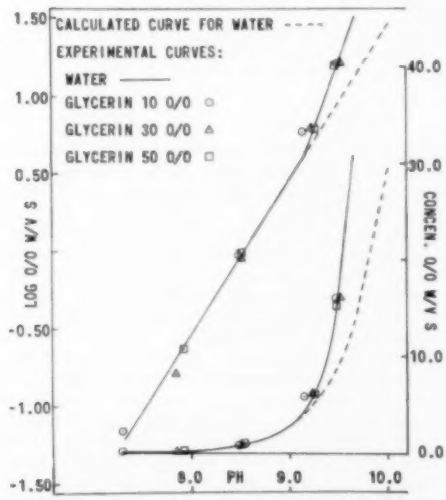

Fig. 3.—Two plots showing the solubility of sodium sulfadiazine in water and in glycerin-water systems at 25° C. as the function of *p*H.

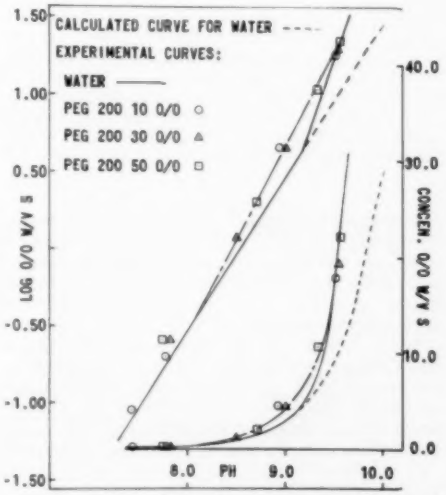

Fig. 4.—Two plots showing the solubility of sodium sulfadiazine in water and in PEG 200-water systems at 25° C. as the function of *p*H.

Figure 1 shows the theoretical and experimental solubility curves.

Solubility of Sulfadiazine Sodium in Other Solvents.—The experimental procedure was exactly the same as above except various concentrations of solvents were substituted for the water. The solvents used were: alcohol, glycerin, propylene glycol, sorbitol solution, polyethylene glycol 200 and polyethylene glycol 400. The solubility curves are presented in Figs. 2–7.

DISCUSSION

If the buffer equation:

$$pH = pKa + \log \frac{\text{salt}}{\text{acid}} \qquad (1)$$

is rearranged as:

$$\log (\text{salt}) = \log (\text{acid}) + pH - pKa \qquad (2)$$

a straight line relationship will exist if log (salt) is

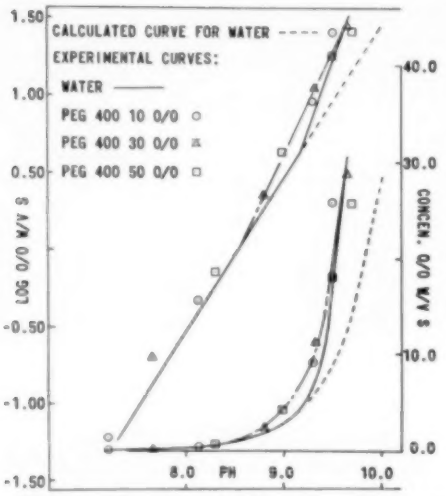

Fig. 5.—Two plots showing the solubility of sodium sulfadiazine in water and in PEG 400-water systems at 25° C. as the function of *p*H.

plotted against the *p*H. Figures 1–7 are plotted in conformity to Eqs. 1 and 2. Both types of plots were utilized to give a more vivid demonstration of the dissolution phenomenon of sulfadiazine sodium at various hydrogen ion concentrations in several solvent systems.

Figure 1 indicates how closely the experimental curve follows the theoretical curve up to a *p*H of approximately 9.2. The reasons for this deviation may be explained on the basis that certain liberties were taken in the use of the buffer equation. It is well known that the use of the buffer e quation is not exact, since in most instances activities are not used and the *pKa* is not the thermodynamic value. The change in the slope (Fig. 1) of the linear curve seems to indicate also that at higher *p*H values complex formation or hydration of the salt occurs. If this is so, then once again the buffer equation would not predict the solubility of the drug above *p*H of approximately 9.2. Recognizing these limitations, however, a study of the experimental data does give a comparison of the relative merits of one solvent over another for the dissolution process of sulfadiazine sodium.

A critical examination of Figs. 1–7, indicates that PEG 200 and PEG 400 were better solvents for the dissolution of sulfadiazine sodium at the *p*H values between 8.3 to 9.5 than the other solvents. The solubility of sulfadiazine sodium in propylene glycol (Fig. 6) follows approximately the solubility curve of the solute in water. There is a slight increase in solubility of sulfadiazine sodium in sorbitol solution (Fig. 2) and in glycerin (Fig. 3) between the *p*H range of 8.8 to 9.4. The use of alcohol (Fig. 7) indicates the effect of this solvent on decreasing the solubility of the solute. In this instance the solubility decreased progressively up to a *p*H of about 9.2 and then increased at the higher *p*H values approaching the solubility in water.

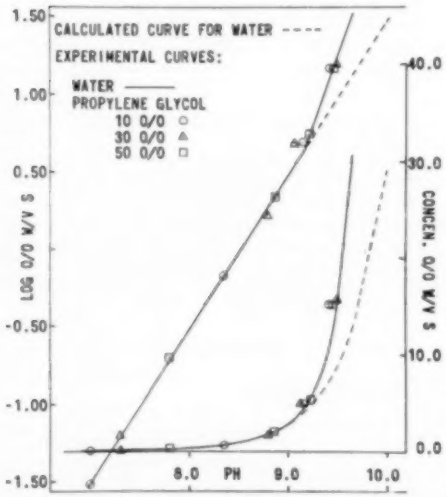

Fig. 6.—Two plots showing the solubility of sodium sulfadiazine in water and in propylene glycol-water systems at 25° C. as the function of *p*H

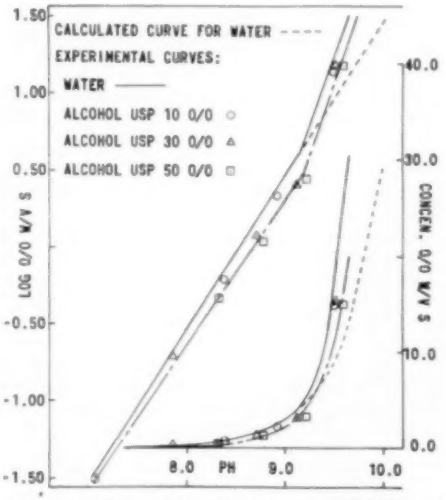

Fig. 7.—Two plots showing the solubility of sodium sulfadiazine in water and in alcohol-water systems at 25° C. as the function of *p*H.

From the experimental evidence presented in this paper it would seem that there is not a great significance among the various solvents to solubilize sulfadiazine sodium. At *p*H values less than 8.0 and at *p*H values more than 9.5, the solubility curves of the drug in the various solvents parallel the solubility curve of the drug in water. In the range of *p*H 8.0 to 9.5, the polyethylene glycols imparted greater solubility to sulfadiazine sodium than the other solvents. Sorbitol and glycerin had the next

greatest effect in solubilizing the drug in the above pH range. The alcohol had a negative effect on solubility of the sulfadiazine sodium at nearly all hydrogen ion concentrations.

There was little effect on the dissolution process when the concentration of solvent was increased from 10 to 50%.

SUMMARY

This study was conducted to ascertain the merits of a number of solvents which have been employed in injectable products for their solubilizing effect on sulfadiazine sodium. Results indicated that the polyethylene glycols had the greatest solubilizing effect while alcohol had the least. In no instance, however, was the magnitude of solubility of such a great nature that it could be considered outstanding compared to water. Further research is being conducted to study the stability of sulfadiazine sodium in several solvent systems at various storage conditions.

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The Chromatographic Separation of Morphine from its Degradation Products*

By JOHN L. LACH, DAHYABHAI M. PATEL, and SEYMOUR M. BLAUG

When solutions of morphine salts undergo prolonged storage a yellow-brown discoloration results due to the partial decomposition of morphine to pseudomorphine and higher oxidation products. These degradation products interfere with the quantitative determination of intact morphine molecules by gravimetric, spectrophotometric, colorimetric, and ion exchange procedures. There are some procedures for estimating the extent of decomposition of morphine salt solutions using these methods. A chromatographic method has been developed for isolating morphine on a partition column and determining it spectrophotometrically after elution. Quantitative recovery was obtained in the presence of pseudomorphine and higher oxidation products.

IN A STUDY of the kinetics of degradation of morphine sulfate in aqueous solution it is necessary to follow the concentration of intact morphine molecules. Although there are numerous methods (1–13) for the quantitative determination of morphine none are satisfactory for the determination of morphine in the presence of its major degradation product pseudomorphine (14–16), and the higher oxidation products formed on the decomposition of pseudomorphine itself (17).

It was the purpose of this investigation to develop an analytical method for the determination of morphine in the presence of its degradation products so that the kinetics of degradation of morphine salt solutions could be studied.

* Received April 30, 1956, from the State University of Iowa, College of Pharmacy, Iowa City.

EXPERIMENTAL

The methods for quantitatively determining morphine range from gravimetric to spectrophotometric procedures. These methods are satisfactory for freshly-prepared solutions of morphine sulfate. Solutions of morphine salts that have undergone prolonged storage develop a yellow-brown discoloration which is attributed to the partial decomposition of morphine to pseudomorphine (14, 15). The color formed and the presence of pseudomorphine interferes with the quantitative determination of morphine in such solutions.

The method developed in this study is based on the isolation of morphine by partition chromatography and its determination in the effluent by spectrophotometric procedures.

According to Balls (8) pseudomorphine may be precipitated from aqueous morphine salt solutions by the addition of excess phosphate buffer pH 6.5. Preliminary investigation of silicic acid partition columns containing an immobile phosphate buffer phase indicated that morphine was partially ad-

sorbed by silicic acid. Consequently, Celite® 535,[1] a diatomaceous earth which is neutral in reaction, was investigated. The success of the chromatographic technique using Celite 535 is evident from the chromatogram shown in Fig. 1. This chromatogram was obtained using a 1 molar phosphate buffer *p*H 6.5 as the internal phase. The concentration of the chloroform-butanol mixture used as the eluant was determined experimentally by liquid-liquid extraction procedures. At this concentration (15% *n*-butyl alcohol in chloroform) morphine in cation form is quantitatively removed from the column whereas pseudomorphine and its higher oxidation products remain behind. If a lower concentration of butanol in chloroform is used, the peak appears late; if a higher concentration is used, small amounts of pseudomorphine appear in the eluate.

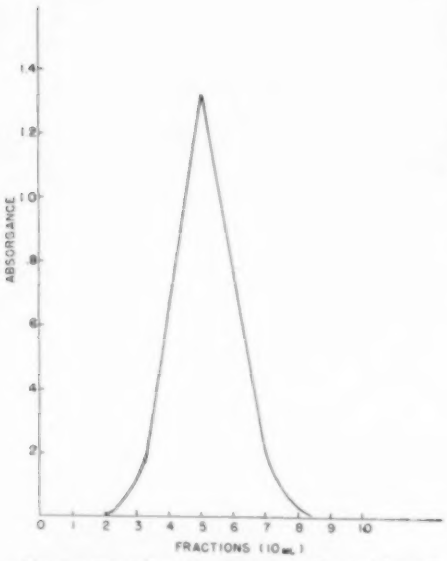

Fig. 1.—Partition chromatogram of morphine sulfate using 15% *n*-butyl alcohol in chloroform as the eluant.

The concentration of morphine in the effluent obtained from the partition column was determined spectrophotometrically at 286 mμ which is the wavelength used for analyzing aqueous solutions. The absorption peak of morphine sulfate in chloroform-butanol solution is 288 mμ as evident from Fig. 2.

Reagents and Apparatus.—Celite® 535, reagent grade *n*-butyl alcohol and chloroform, 1 *M* phosphate buffer *p*H 6.5, recrystallized morphine sulfate, and pseudomorphine sulfate. Pyrex® glass chromatographic columns 45 cm. long and 2 cm. in diameter with glass plungers to pack the columns. Beckman Model DU spectrophotometer equipped with a photomultiplier and 1-cm. silica cells.

Preparation of Column.—The columns are prepared by thoroughly mixing 15 ml. of the phosphate buffer with 15 Gm. of Celite® 535. A slurry is made of this by adding 15 ml. of butanol-chloroform mixture. The mixture is then transferred to a column with frequent packing. The slurry should be packed so that the flow rate adjusts to approximately 2–3 ml. per minute.

Preparation of Pseudomorphine Sulfate.—Pseudomorphine sulfate was prepared by the mild oxidation of morphine in alkaline solution with $K_3Fe(CN)_6$. The pseudomorphine formed was purified by dissolving it in sodium hydroxide solution and reprecipitating with CO_2. The sulfate was prepared by dissolving the base in glacial acetic acid and precipitating it as the sulfate with sulfuric acid. After recrystallization from water and drying *in vacuo* the sulfate melted and decomposed above 327°.

Anal.—Calcd. for $C_{34}H_{36}N_2O_6 \cdot H_2SO_4 \cdot 2H_2O$: C, 58.11; H, 6.02; N, 3.98. Found: C, 59.97; H, 6.08; N, 3.93.

Procedure.—Exactly 5 ml. of an aqueous solution of morphine sulfate was pipetted onto 4 Gm. of Celite® 535 contained in a beaker. The two were thoroughly mixed, then transferred quantitatively to the column using 2 × 5-ml. portions of the chloroform-butanol mixture to rinse the beaker. The slurry was tightly packed on the surface of the column with a glass plunger which was rinsed with some chloroform-butanol mixture before removing from the column. The first 20 ml. of eluate was discarded, then 100 ml. was collected in a volumetric flask and its absorbance was measured using as a blank a chloroform-butanol mixture that had been passed through a column containing no morphine sulfate. Recovery data are shown in Table I.

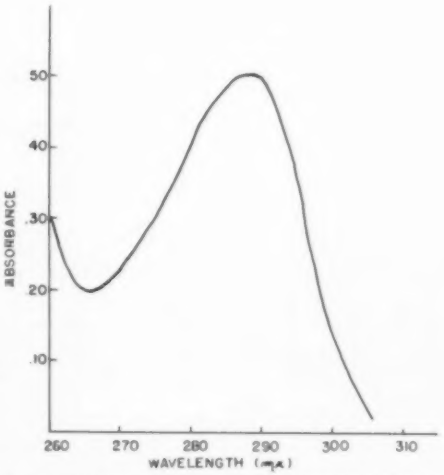

Fig. 2.—Absorption spectrum of morphine sulfate in 15% *n*-butyl alcohol in chloroform.

The same procedure as above was followed using freshly prepared solutions of pseudomorphine sulfate and pseudomorphine solutions which had been

[1] Celite 535 was supplied by the Johns-Manville Co.

partially decomposed by autoclaving so that a distinct yellow-brown discoloration was present. One hundred and fifty milliliters of eluate was collected from each column and analyzed spectrophotometrically. The absorbance was the same as that obtained by passing 150 ml. of chloroform–butanol mixture through a column containing no pseudomorphine. The yellow-brown color of the partially decomposed samples did not appear in the eluate.

TABLE I.—RECOVERY OF MORPHINE SULFATE FROM CELITE® 535

Theoretical, mg.	Recovered, mg.	Recovery, %
7.23	7.22	99.86
7.23[a]	7.17	99.17
7.24	7.29	100.69
7.45	7.50	100.70
8.86	8.92	100.67
8.86[a]	8.83	99.66
10.76	10.75	99.90
10.86	10.81	99.54
10.86[a]	10.82	99.63
11.36	11.30	99.70

[a] Pseudomorphine and oxidized pseudomorphine solutions were added to these solutions before chromatographic analysis.

DISCUSSION

Although there are many methods for the quantitative determination of morphine sulfate in aqueous solution these methods are not satisfactory for a kinetic study since they do not differentiate between intact morphine molecules and the oxidation products of morphine. The method developed in this study is based on the isolation of intact morphine molecules by partition chromatography. This procedure is based on the difference in solubility of morphine sulfate and pseudomorphine sulfate and its higher oxidation products in an immobile phase *p*H 6.5 phosphate buffer. The morphine sulfate is soluble in the immobile phase and can be eluted with a mobile organic solvent phase. The recovery data shown in Table I indicate that recovery is quantitative even in the presence of pseudomorphine sulfate and its higher oxidation products. Results of a kinetic study of morphine salt solutions now under way in our laboratories will be presented in a future communication.

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Determination of Reserpine*

By C. R. SZALKOWSKI and W. J. MADER†

A rapid and specific method for the analysis of reserpine was needed. It was observed that a fluorescent, greenish-yellow color was obtained by treating an alcoholic solution of reserpine with dilute sulfuric acid and sodium nitrite. This reaction has been adapted to the quantitative determination of reserpine. The method is applicable to the determination of reserpine in tablets, solutions, and elixirs. Similar alkaloids such as alstonine, rauwolscine, sarpagine, raunescine, deserpidine, and yohimbine do not interfere.

RESERPINE has become an important agent in the treatment of hypertension and other maladies. As therapeutic preparations containing crystalline reserpine in various dosage forms become more numerous, the problems of control also increase and the need for specific assay procedure becomes more pressing.

The ultraviolet absorption characteristics of reserpine have been utilized in assay procedures together with separation techniques which provide for isolation of the reserpine (1–3).

Poet and Kelly (4), reported a fluorometric procedure for the determination of reserpine based on heating samples containing reserpine in a boiling water bath in the presence of sulfuric acid or a mixture of sulfuric and selenious acids. McMullen, *et al.* (5), tested the effect of various acids on reserpine and reserpic acid and found

* Received April 16, 1956, from Chemical Control Division, Merck and Company, Inc., Rahway, N. J.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 13, 1956.
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that a mixture of sulfuric and hydrochloric acids gave the most stable and reproducible results. Dechene (6) reported that the fluorescence of solutions under ultraviolet light was increased by heating with hydrogen peroxide, and was quantitatively related to its concentration.

Reserpine may also be determined colorimetrically by the formation of a chloroform-soluble dye complex (7) and by reaction with vanillin (8).

The authors have observed that reserpine, reserpic acid, methyl reserpate, and rescinnamine, when treated with dilute sulfuric acid and sodium nitrite, give greenish-yellow, fluorescent solutions which exhibit an absorption maximum at 388–390 mμ. The absorbances when developed under identical conditions conform to Beer's law and are suitable for the determination of reserpine.

Since reserpine is a weakly basic substance, it can be separated from stronger alkaloids by extraction from acidic solutions with chloroform. This characteristic is utilized to separate reserpine from reserpic acid.

It has been established that alkaloids such as alstonine, rauwolscine, sarpagine, yohimbine, and deserpidene do not give this reaction. A number of formulation ingredients such as acetylsalicylic acid, amphetamine, caffeine, mepiperphenidol, phenobarbital, pipradol, and others have been tested and do not show this reaction. These considerations led to the development of the method presented for the determination of reserpine.

PROPOSED PROCEDURE FOR ANALYSIS

Reagents.—(Unless otherwise indicated, all reagents used are reagent grade.) Anhydrous methyl alcohol; chloroform; sodium bicarbonate, 1% aqueous solution; citric acid, 2% aqueous solution; sulfuric acid, 0.5 *N* and 1.0 *N* solutions; and sodium nitrite, 0.30 Gm. dissolved in 100 ml. water, prepared daily.

Reference Standard Reserpine.—Crystalline reserpine, assayed by phase solubility analysis using methyl isobutyl ketone (sol. 6 mg./Gm.) is used as reference standard.

Standard Reserpine Solution.—Dissolve 25.0 mg. of reserpine standard in about 35 ml. of hot methyl alcohol, protecting the solution from direct light, cool and dilute with anhydrous methyl alcohol to make exactly 100 ml. (This standard solution, when stored in tightly-stoppered brown glass bottle is stable for at least a week. Working standards are prepared by further dilution with methyl alcohol.)

Apparatus.—Absorbance measurements were made with a Cary Recording Spectrophotometer, Beckman DU or Beckman Model B Spectrophotometer.

Procedure.—Prepare a methyl alcohol solution of the substance to be tested containing 0.010 to 0.020 mg. reserpine per ml. Protect the solution from direct light. Pipet 5.0 ml. of the sample solution into a large test tube, add 3.0 ml. of 0.5 *N* sulfuric acid and mix. Then add 2.0 ml. of 0.3% sodium nitrite solution, mix, and let stand for one hour. Determine the absorbance of the solution in a suitable spectrophotometer set at 390 mμ against a blank prepared from 5.0 ml. anhydrous methyl alcohol, 3.0 ml. of 0.5 *N* sulfuric acid, and 2.0 ml. of water.

Preparation of Standards.—Prepare standards containing 50, 100, and 150 μg. reserpine per 5.0 ml. of methyl alcohol. Pipet 5.0 ml. of the standard solutions into large test tubes and proceed as for unknown. The standards are run simultaneously with the unknown.

EXPERIMENTAL

Spectral Characteristics.—The absorption spectrum of reserpine in anhydrous methyl alcohol as determined on a Cary recording spectrophotometer using 1-cm. cells is shown in Fig. 1, curve A. A

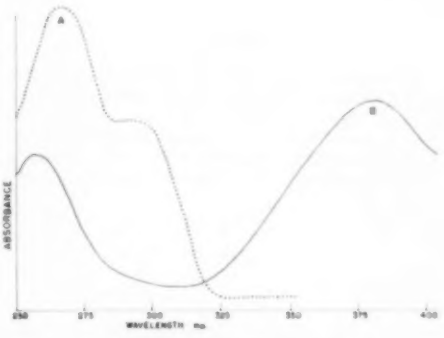

Fig. 1—Absorption curves.

maximum at 268 mμ and a secondary maximum at 296 mμ are indicated. The identity of the reserpine may be established by determining the ratio of the absorbances at 268 mμ and 295 mμ. A_{268}/A_{296} = 1.63 ± 0.05. Curve B shows the absorption curve obtained after 0.3 mg. reserpine in 5.0 ml. of anhydrous methyl alcohol was treated with 3.0 ml. of 0.5 *N* sulfuric acid and 2.0 ml. of 0.3% sodium nitrite solution under the conditions of the Procedure. A maximum absorption at 390 mμ is obtained. This maximum was obtained with acids other than sulfuric in which case the intensity of the absorption varied with the acid used. Sulfuric acid produced the greatest intensity followed by phosphoric, hydrochloric, nitric, formic, and acetic acids.

Effect of Sulfuric Acid.—Figure 2 shows the effect of the normality of the sulfuric acid added on the absorbance of the colored solution. In this series of experiments, 5.0 ml. of methyl alcohol solution containing 0.18 mg. of reserpine was treated with 3.0 ml. of sulfuric acid of specified normality and 2.0 ml. of 0.3% sodium nitrite solution under the

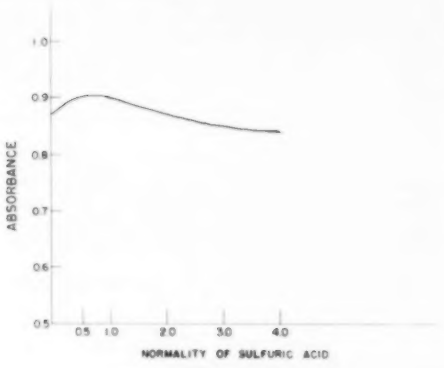

Fig. 2—Effect of H_2SO_4.

conditions of the Procedure. The absorbance of the colored solution remained constant and stable for normalities of 0.4 to 1.0 and was stable for at least six hours. The absorbance and stability decreased when the sulfuric acid added was of a normality greater than one. For convenience 0.5 *N* sulfuric acid was chosen.

Effect of Sodium Nitrite.—In this series of tests, 5.0 ml. of stock solution containing 0.18 mg. of reserpine per 5.0 ml. of methyl alcohol was treated with 3.0 ml. of 0.5 *N* sulfuric acid and 2.0 ml. of sodium nitrite solution containing 1.0 to 30 mg. of sodium nitrite per ml. under the conditions of the Procedure. The spectral characteristics of the greenish-yellow color were not affected by the amount of sodium nitrite added. The absorbance and stability decreased when the amount of sodium nitrite added exceeded 8 mg. (Fig. 3).

Effect of Time.—This effect was studied by treating separate 5.0-ml. portions of methanol stock solution containing 48.2, 96.5, and 193 μg. of reserpine, respectively, with 3.0 ml. of 0.5 *N* sulfuric acid and 2.0 ml. of 0.3% sodium nitrite solution at room temperature. The spectral characteristics and intensities of the solutions were determined at different time intervals. The results are shown in Table I. Maximum intensity was reached in sixty minutes and was constant for at least six hours.

TABLE I.—EFFECT OF TIME ON ABSORBANCE

Minutes	48.2 γ	96.5 γ	193.0 γ
5	0.210	0.400	0.810
10	0.220	0.420	0.860
15	0.220	0.430	0.870
20	0.225	0.445	0.880
30	0.225	0.455	0.890
45	0.225	0.460	0.905
60	0.225	0.460	0.920
120	0.225	0.460	0.915
180	0.225	0.460	0.920
240	0.225	0.460	0.920
300	0.225	0.460	0.920

Calibration Curve.—A plot of the absorbances against concentration of reserpine was found to be linear and passed through the origin. Beer's Law is obeyed over a suitable range of concentration, 0 to 200γ. Table II gives the absorbance obtained at 390 mμ at three levels of reserpine.

TABLE II.—REPRODUCIBILITY OF ABSORBANCE AT THREE CONCENTRATIONS AT 390 Mμ

	48.2 γ	96.5 γ	193.0 γ
	0.230	0.450	0.900
	0.225	0.460	0.910
	0.235	0.460	0.920
	0.230	0.455	0.910
	0.220	0.445	0.900
	0.225	0.460	0.915
	0.230	0.460	0.920
	0.220	0.455	0.915
	0.227	0.456	0.910
Av. dev.	±0.004	±0.004	±0.006

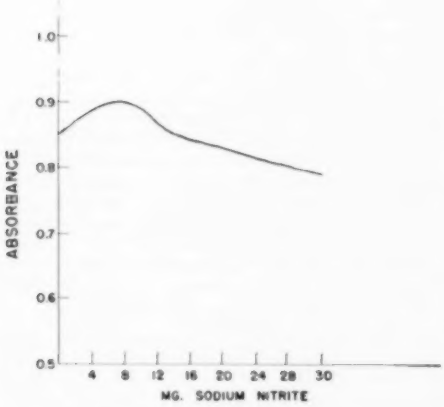

Fig. 3—Effect of sodium nitrite.

Procedure for Assay of Tablets.—The procedure used for the determination of reserpine in tablets follows:

Weigh a counted number of not less than 20 reserpine tablets, and reduce them to a fine powder. Weigh accurately a quantity of the fine powder equivalent to 0.5–1.0 mg. reserpine into a 50-ml. stoppered centrifuge tube. Add 10 ml. of 2% citric acid solution and shake for three minutes. Add 10 ml. of chloroform, shake for three minutes, and centrifuge. Draw off the chloroform layer into another 50-ml. centrifuge tube by means of a syringe. Repeat the extraction with two additional 10-ml. portions of chloroform. To the combined chloroform extracts add 10 ml. of 1% sodium bicarbonate solution, shake for two minutes, and centrifuge. Draw off the aqueous layer and discard. Filter the chloroform extract into a 100-ml. beaker, washing

the filter and tube with chloroform. Evaporate the combined chloroform extracts on a steam bath. Add 25 ml. of warm anhydrous methyl alcohol to dissolve the residue in the beaker. Cool and transfer to a 50-ml. volumetric flask and make up to volume with methyl alcohol.

Pipet 10.0 ml. of the methyl alcohol solution into a large test tube, add 3.0 ml. of 1 *N* sulfuric acid and mix. Then add 2.0 ml. of sodium nitrite solution, mix, and let stand for one hour. Filter through a dry 9-cm. Whatman No. 42 filter paper. Determine the absorbance of the filtered solution at 390 mμ in a spectrophotometer set against a blank prepared from 10.0 ml. methyl alcohol, 3.0 ml. of 1 *N* sulfuric acid and 2.0 ml. water.

by the vanillin test (8). The results are compared in Table III. Good agreement was obtained between the proposed method, phase solubility analysis and ultraviolet analysis.

The recovery of reserpine from known mixtures of reserpine, lactose, starch, and magnesium stearate using the proposed procedure for tablets ranged from 97 to 101%.

Table IV lists the data obtained in applying the proposed procedure for tablets to a series of commercial samples of reserpine tablets. The same extraction procedure was used in comparing the proposed method with the ultraviolet method for these tablets.

The proposed method was applied to tablets

TABLE III.—ASSAY RESULTS FOR RESERPINE, PER CENT RESERPINE

No.	Brand	Proposed Method	A_{268}/A_{296}	U. V. Analysis —Calculated from, %— A_{295}	A_{268}	Phase Solubility	Vanillin Method
C-1	A	95.0	1.63	94.0	94.0	94.7	107.0
B-10	B	96.9	1.63	98.3	98.3	..	..
C-20	C	93.9	1.61	94.1	92.9	..	..
D-23	D	94.5	1.60	93.1	91.1	..	..
E	E	93.0	1.60	94.2	93.0	..	..
F	F	97.0	1.62	97.3	97.2	..	..
P	G	96.0	1.63	95.7	95.7	..	103.0
PF	H	96.8	1.63	96.6	96.6	..	93.0
M-1	I	95.6	1.61	96.0	95.3	96.0	96.0
M-2	I	95.5	1.61	94.8	94.0	..	99.0
M-3	I	95.5	1.63	95.3	95.5	95.0	..
M-4	I	95.2	1.62	98.5	98.3	94.7	97.0
M-5	I	95.0	1.63	95.0	95.0	95.0	..
M-6	I	96.0	1.64	96.2	97.4	..	..
M-8	I	96.9	1.65	94.5	95.7	96.0	..
M-9	I	96.7	1.63	96.7	97.1	95.8	104.0
M-10	I	94.7	..	..	..	96.0	96.0
M-11	I	87.4	..	..	..	88.4	..
P-2	G	92.5	..	..	..	93.0	103.0
C-2	A	97.2	..	..	..	97.0	..

Preparation of Standards.—Prepare standards containing 100.0, 150.0, and 200 μg. reserpine per 10 ml. methyl alcohol. Pipet 10.0-ml. aliquots into separate tubes and run simultaneously in the same manner as the unknown.

Procedure for Injections and Elixirs.—Dilute a volume of the sample equivalent to 0.5 to 1.0 mg. reserpine with 25 ml. of water in a separatory funnel. Extract the diluted solution with 20 ml. of chloroform. Draw off the chloroform layer into another separator and repeat the extraction with 3 additional 10-ml. portions of chloroform. Shake the combined chloroform extracts with 15 ml. of 1% sodium bicarbonate. Allow to settle and then draw off the chloroform into a 100-ml. beaker. Evaporate the chloroform on a steam bath. Take up the residue in 25 ml. of warm methyl alcohol. Cool and make to 50 ml. with methyl alcohol.

Determine the absorption of 10-ml. aliquots of this solution as described in the Procedure for tablets.

DISCUSSION

Different manufacturers' lots of reserpine were analyzed by the proposed method, a phase solubility analysis method (9), ultraviolet analysis, and which contained reserpine compounded with other active ingredients. The results are tabulated in Table V. In the case of samples 10, 14, and 17, the method fails due to the presence of mannitol hexanitrate. Mixtures of reserpine, lactose, and mannitol hexanitrate were prepared and assayed. The recoveries at best were 60% of the added amounts of reserpine. Rutin gives a yellow color in alcoholic solution with nitrite and sulfuric acid, but it does not interfere in the test because it cannot be extracted from an aqueous solution with chloroform. In the case of sample 13, the tablets contained reserpine, acetylsalicylic acid, phenacetin, caffeine, and a yellow dye. The chloroform extract of these tablets was a yellow solution, and the color could not be extracted with sodium bicarbonate solution.

Injection solutions and elixirs were also subjected to analysis, and the results obtained are listed in Table VI.

SUMMARY

1. The fluorescent greenish-yellow color obtained by treating an alcoholic solution of reserpine with dilute sulfuric acid and sodium nitrite has been used for its quantitative deter-

TABLE IV.—ANALYSIS OF TABLETS

No.	Brand	Label Claim γ/Tablet	Proposed Method γ/Tablet	U. V. Analysis A_{330}/A_{350}	U. V. Analysis γ/Tablet A_{330}	U. V. Analysis γ/Tablet A_{350}
R-20	C	1000	1001	1.63	1002	1002
G	B	250	254	1.62	246	244
H	A	250	247	1.60	240	234
1	C	250	239	1.62	244	242
2	D	300	274	1.63	261	259
3	E	250	234	1.54	257	242
4	F	1000	908	1.62	912	904
4A	F	250	238	1.75	243	257
5	G	250	239	1.89	247	281
6	H	250	244	1.60	240	234
20	J	1000	977	1.65	959	971
21	J	100	93.0	..	..	..
22	I	100	91.0	1.68	90.8	94.1
23	I	100	95.4	1.65	96.0	97.0
24	I	1000	961	1.66	963	970

TABLE V.—ASSAY OF TABLETS CONTAINING OTHER ACTIVE INGREDIENTS

No.	Brand	Label Claim γ/Tablet	Active Ingredients	Reserpine Found γ/Tablet
7	K	250	Reserpine, phenobarbital calcium	247
8	L	100	Reserpine, protoveratrine A & B	96
9	M	250	Reserpine, pipradol	237
10	N	100	Rutin, ascorbic acid, reserpine, mannitol hexanitrate, theophylline	48
12	C	100	Hydralazine, reserpine	64
13	J	100	Acetylsalicylic acid, phenacetin, reserpine, caffeine	43
14	J	100	Reserpine, rutin, mannitol hexanitrate	54
15	J	100	Reserpine, *d*-amphetamine	98
16	J	100	Pyrilamine, phenylpropanolamine, reserpine	100
17	J	100	Reserpine, rutin, mannitol hexanitrate	73
18	J	100	Reserpine, vinbarbital sodium	94
19	J	250	Reserpine, mepiperphenidol	234

TABLE VI.—ASSAY OF ELIXIRS AND INJECTION SOLUTIONS

No.	Brand	Active Ingredients	Label Claim	Reserpine Found
25	J-Injection	Reserpine	250 γ/ml.	238 γ/ml.
27	J-Elixir	Reserpine, vitamin B_1, vitamin B_{12}, B_6, nicotinamide	100 γ/15 ml.	108 γ/15 ml.
26	F-Injection	Reserpine	250 γ/ml.	240 γ/ml.
28	H-Elixir	Reserpine	50 γ/ml.	53 γ/ml.

mination. Similar alkaloids such as alstonine, rauwolscine, sarpagine, raunescine, deserpidine, and yohimbine do not interfere.

2. A method is described for the extraction of reserpine from tablets, solutions, and elixirs. The methods work in the presence of such formulation ingredients as acetylsalicylic acid, amphetamine, caffeine, mepiperphenidol, phenobarbital, phenylpropanolamine, pipradol, pyrilamine, vinbarbital, protoveratrines A and B, thiamine, pyridoxine, nicotinamide, and vitamin B_{12}.

3. Formulation ingredients such as hydralazine, mannitol hexanitrate, acetophenetidin, and theophylline inhibit the color formation.

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Production of Water-Soluble or Dispersible Pills by the Drop Method

By SKULI BJORNSSON and ORVILLE H. MILLER

Preparation of pills by the drop method provides a dosage form of very uniform weight. This method, previously limited to fatty materials, can be applied to water-soluble or dispersible substances. The effect on pill weight of variations in orifice dimensions, liquid level, drip rate, and temperature of melt was determined. These must be maintained constant to provide pills of uniform weight. Water-soluble pills containing sodium phenobarbital were prepared in which variation in weight did not exceed 2 per cent. Pharmaceutical applications of this procedure warrant further study.

ORAL DOSAGE FORMS such as compressed tablets and capsules have certain limitations, for example: the accuracy in dosage leaves much to be desired (see (1) and Fig. 1), and the requisite processing may prove deleterious to unstable agents. Here the so-called drop method seems applicable to the preparation of low dosage products, in the form of pills of very uniform weight (see Fig. 2) and with increased stability because of less handling (2–5). By the use of the drop method the active agents are dissolved or emulsified in a material, or a mixture of materials, with a suitable congealing point. In molten or liquefied state they are allowed to form into drops and fall through an indifferent liquid of specific gravity slightly lower so the formed drops fall very slowly through the liquid. During the fall they assume a completely round spherical form due to the surface tension of the melt. The temperature of the liquid is adjusted such that the drops congeal on their passage through the liquid to a solid pill-shaped body. The drop method offers several advantages: first, its ease of production; secondly, the process diminishes the exposure of the active agents to air and minimizes their eventual oxidation during processing; lastly, uniform and exact dosage forms are obtained. These advantages are especially valuable where highly active or costly materials are concerned. Literature on the subject is limited, as the process of the drop method seems to have been overlooked by the pharmaceutical profession, even though it has been utilized by the chemical industry for some time in producing lead grains, pellets of alkali, and other formed materials. The first report available regarding the use in pharmaceutical practice is in a patent: "Apparatus and method for transforming molten material into a form of solid round bodies such as pills" (3). It was issued in 1933 to Ferrosan, a pharmaceutical company in Copenhagen, Denmark. The method

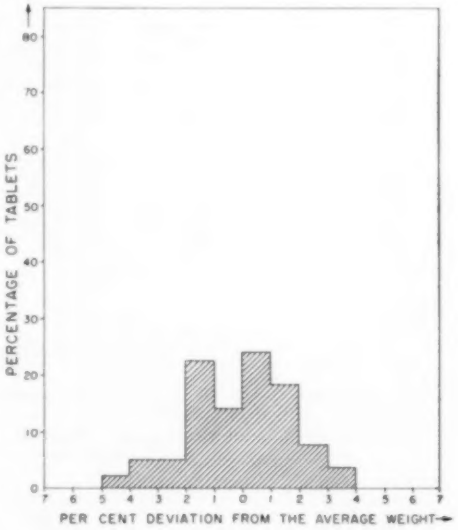

Fig. 1.—Variation in weight of stilbestrol implant tablets. 3,900 per hour. Av. wt., 15 mg. (Hansen, G. (1).)

and apparatus were employed in producing A and D vitamin pills with a base of fats or hydrogenated oils, and diluted ethanol (for example) was used as a cooling liquid. Another patent has been applied for (6) by Lovens kemiske Fabrik, also in Denmark. It differs from the first in that the melted fatty substances are introduced from the bottom of the cooling container. A medium for cooling with a higher specific gravity than the molten substance is employed,

Received March 2, 1956, from the School of Pharmacy, University of Southern California, Los Angeles.
This paper is adapted from the prize-winning manuscript submitted by Skuli Bjornsson in the Western Region, 1955 Lunsford Richardson Pharmacy Awards competition.
This paper is part of a thesis submitted by Skuli Bjornsson to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

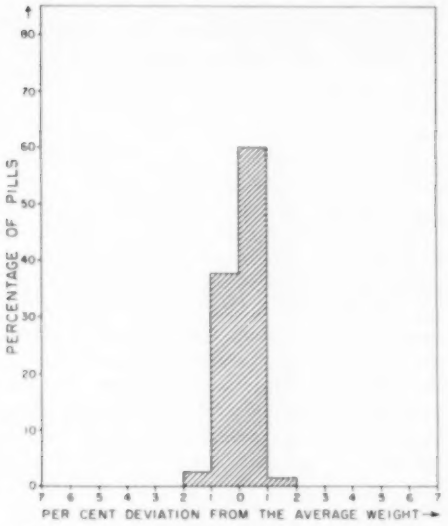

Fig. 2.—Variation in weight of A–D vitamin pills processed by the Drop Method (Machine—Termansen, J. B. (2)). 6,000–9,000 per hour. Av. wt., 70.5 mg.

which causes the drops formed to ascend to the surface. Sandell (4) in his report of a simple arrangement to produce vitamin A pills by the drop method recorded the first published literature on the subject. He pointed out that the drop weight depends on the size of the opening of the glasspipe used (the inside diameter of the drainer), the surface level in the drainer, the temperature, and the drip rate. He used hydrogenated peanut oil with a melting point of approximately 41°. Temperature of the base containing the dissolved A vitamin was kept at 60°, and the cooling liquid (a 65% ethanol-water mixture) at room temperature. With this simple arrangement (Fig. 3) he obtained, with a drip rate of 30 drops a minute, fairly good accuracy in dosage with pills of 78 mg. average weight. The average difference in weight was about 0.7%, and maximum difference of approximately 2%. He suggested promising results for preparing pills by this method in practice. A later report from Sandell and his co-worker Ernerfeldt (5) pointed out several difficulties in the regulating system used in the arrangement Sandell previously had reported (4). First, the small canals (as within a glass stopcock) could not be used because of the tendency to clog by the congealing mass and, secondly, the drip rate was not satisfactorily controlled. As changes of the surface level cause great variations in the drip rate and thus the weight of the drops, a double siphon system to obtain a constant surface level of the dripping substance was suggested. The drip rate could be regulated by changing the surface level between the interior and exterior siphon systems. Constant temperature, they reported, is extremely important for the uniform weight of the drops and to retain the shape of the pill. It was demonstrated that the optimum temperature of the melted substance was 59° and that below 55° the pills did not obtain a completely round form in the cooling liquid at room temperature. The diameter of the drainer and the shape of the opening was shown to be of great importance for constant size and weight of the drops, and the optimal fall of the drop was found to be 2 cm. In experimental runs with this improved arrangement the uniformity of the product was excellent, with a standard difference of only 0.6%. Hydrogenated peanut oil was used as a base (m.p., 38–40°), the diameter of the drainer 7 mm., temperature 58°, and drip rate 90 drops per minute. Termansen (2) reported on his work in designing a machine, the so-called "Piluterm"[1], of the pumping type which measures the exact amount of substances to be dripped, thus eliminating the surface level problem and the forming of pills by nonmeasured dripping. This machine can be easily adjusted for different sizes of pills, and regulated in drip rate. It is a great improvement in procedure and expands the pos-

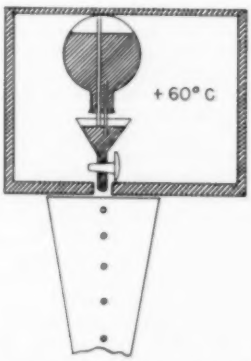

Fig. 3.—Arrangement used by Sandell, E. (4). The apparatus consisted of a constant temperature oven where the fat mixture was kept in a molten state and dripped through the air inlet at the bottom into the cooling liquid. The size of the drops depended on the thick-walled glasspipe used as the drainer (6.5 mm. i. d. and 12.0 mm. o. d.), the height of surface level, and the drip rate.

[1] Now manufactured in Denmark and available in the U. S. from Pharmaceutical and Chemical Industry Supply Corp., Division of American Roland Corporation, 16 Hudson Street, New York 13, N. Y.

sibilities of use for the drop method as a whole. Hydrogenated soybean oil was used as the base for the A-D vitamin pills, and diluted ethanol was the cooling liquid. Termansen showed that with a constant dripping surface the size of the drop will also depend on the surface tension, which will be influenced by temperature fluctuations and by small variations in the composition of the mixture of fatty substances. Furthermore, the size of the drop will be directly affected by the temperature alterations through their influence on the specific gravity of the mixture of fatty substances. Finally, the drip rate has a considerable influence on the size of the drop, as this is increased by the drip rate. These few available reports where the drop method has been applied lack concrete information of the behavior of drops under different physical conditions. The role of physical changes is mentioned, but there is no evidence shown of experimental work performed which can substantiate the statements. The only comparative study found in the literature on drop size and uniformity was published by Adler (7), but it was performed with water, benzene, methanol, and ethanol at room temperature. He points out that the drop weight is a function of the drip rate and the diameter of the drainer. A study of drop behavior in the actual practice of the drop method seems to be necessary to establish concrete figures on drop weight in relationship to the surface level, drip rate, diameter of the drainer, thickness of walls of the drainer, temperature, and the height of the fall of the drops through the air until they have penetrated the surface of the cooling liquid. Such work should also utilize the same base materials as intended for actual practical use. The base used in the production of pills by the drop method has until now consisted of hydrogenated oils or fats with a melting point of approximately 40°, products commonly employed in the oleomargarine industry (2–6). Such fatty bases have the disadvantages that only oil-soluble or oil-miscible materials can be employed as active ingredients, and that the pills formed are neither soluble nor dispersible in water, so that the absorption in the body is delayed. When the addition of other than oil-soluble or oil-miscible materials was desired, they were added during the coating process. In a bulletin (8) from the Danish Apothecary Control Laboratories in Copenhagen, Denmark, a procedure is reported on producing A and D vitamin pills with the addition of ascorbic acid, also combinations with B vitamin materials. This procedure has been used to produce first a core of a pill by the drop method (2), containing A and D vitamins. The core or pill is then coated with the various other vitamin materials and lastly with a colored sugar coating. The stability of such preparations has been reported to be excellent. This method is not only cumbersome, but also undesirable for highly active ingredients because of difficulties with exact dosage. These disadvantages suggested the investigation of other materials to prepare a base, or bases, which were soluble, or dispersible in water. Such bases would provide for the faster release of active materials in the body, and possess solvent action for water-soluble medicinal agents. Such a study could open up new aspects in manufacturing pharmaceutical preparations in dosage form intended for oral use and soluble pills for injections. Its possibilities are greatest where highly active and sensitive materials are used in low dosage form, such as alkaloids, hormones, and others. The reduction in and ease of handling the materials promises higher stability for the finished preparations. The process is simple and can be employed without the need of costly equipment. It could, therefore, be utilized in the practical, daily routine of the retail pharmacy, but it would especially be useful for hospital pharmacies and the pharmaceutical industry. Its economy and ease of production also make it an appealing method for use in producing cores or small pills, which can be further coated with active materials, if required.

EXPERIMENTAL

Recent developments in the chemical field have produced many nontoxic materials, both water-soluble and water-dispersible, that may be adapted to preparation of pills by the drop method. In addition to the pill base which must be of a suitable consistency and melting point, cooling liquids and often solvents for removing cooling liquids are required. Small amounts of solvents for active ingredients, i.e., water, glycerin, propylene glycol, may also be incorporated in the pill base.

The alcohol-water cooling liquids previously used for the fatty bases would not be appropriate for water-soluble and dispersible bases. Various oils and water-immiscible solvents may be used. Volatile solvents such as ether, petroleum ether, isopropyl alcohol, etc., would be required to remove oils used as cooling fluids. One important factor to be considered when organic materials are used as cooling liquids is that their heat conductivities are considerably inferior to those of water or mixtures of water and alcohol. Therefore, it is necessary to use these liquids at lower temperatures than would otherwise be indicated for the water-alcohol mixtures.

In order to evaluate and compare the effects of temperature, diameter of orifice (drainer), drip rate, and variations in liquid level on the weight of

pills formed from fatty materials such as those previously used, with the effects on water-soluble bases, and to establish a quantitative measurement of these effects, a suitable base material of each type was used in this study. Hydrogenated soybean oil (melting point 38°) and polyethylene glycol 4000 (Carbowax® 4000), a water-soluble base material, were selected after a trial of a number of possible materials. These substances readily form pills in the experimental apparatus and no difficulty was encountered in obtaining suitable cooling liquids in which they were insoluble.

A simple experimental apparatus was utilized for the evaluation of the several factors on weight of the pills. This apparatus is described in Fig. 4.

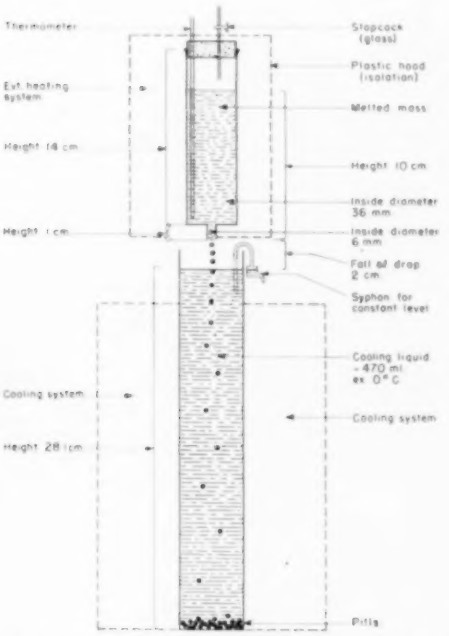

Fig. 4.—Apparatus (experimental) for preparing pills by the Drop Method.

It consists essentially of a container with a dripping outlet on the bottom with the upper end closed with a stopper through which passes an adjustable air vent to control the rate of flow. An external heating system (infrared lamp) and shielding was utilized to maintain a constant temperature in the molten mass. A container of cooling liquid was used to catch the molten droplets. The temperature of the cooling liquid itself was adjusted by immersion in a suitable water bath. This apparatus was used without the cooling liquid and bath in portions of this study where it was not required. The volume of molten base was sufficient to adequately maintain a uniform liquid level during the formation of the small number of pills required for the experiments. Although not shown in Fig. 4,

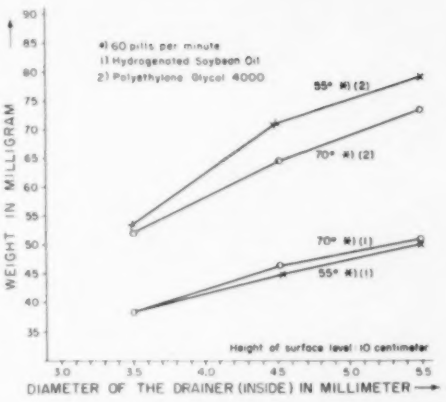

Fig. 5.—Influence of the diameter of the drainer on the weight of the pills produced by the Drop Method.

the dripping orifice was interchangeable to permit studies on the effect of orifice dimensions.

The effect of the following experimental conditions on drop weight were studied: The drip rate was kept constant at 15, 30, 60, 90, or 120 drops per minute. The temperature was maintained at 55, 60, 65, or 70°. The surface level of the molten material was maintained at 5, 10, 15, or 20 cm. above

TABLE I.—EFFECT OF ORIFICE DIAMETER AND WALL THICKNESS OF DRAINER ON DROP WEIGHT OF MOLTEN POLYETHYLENE GLYCOL 4000 (55°)

Inside Diameter, mm.	Outside Diameter, mm.	Weight of Average Drop, mg.
1.6	2.2	27
2.0	3.0	35
2.0	17.0	62
2.8	19.5	73
3.5	5.3	58
5.5	7.5	81

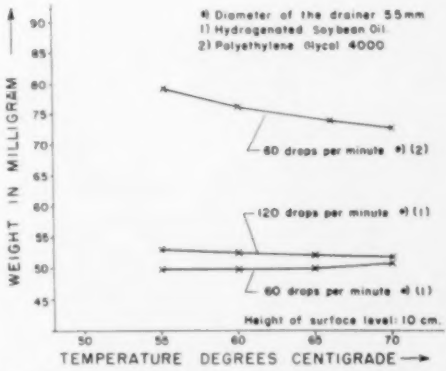

Fig. 6.—Effect of temperature on the weight of the pills produced by the Drop Method.

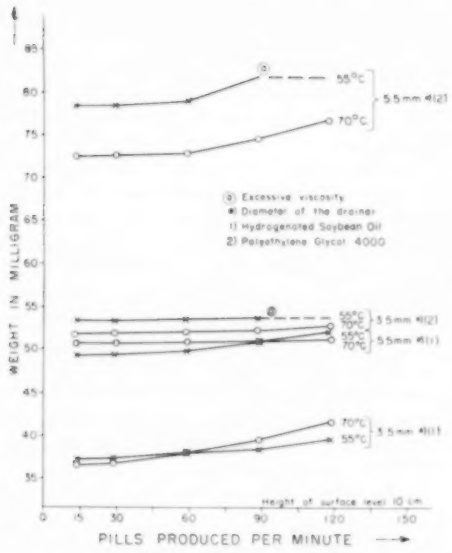

Fig. 7.—Effect of the drip rate on the weight of pills produced by the Drop Method.

the orifice. For each test 50 pills were collected in tared beakers and weighed.

The effect on the weight of pills or drops of molten polyethylene glycol 4000 produced by changes in the diameter of the orifice and the thickness of the orifice wall are shown in Table I.

An additional study was made on the effect of drainer orifice diameter at constant wall thickness on both molten polyethylene glycol 4000 and hydrogenated soybean oil at temperatures of 55 and 70°. Results are shown in Fig. 5.

Using a constant orifice and surface level the average drop weight was determined for the two materials at temperatures of 55, 60, 65, and 70°. The effect of temperature on drop weight is shown in Fig. 6.

Since the drip rate was reported to affect drop size, this was kept constant in the preceding experiments. The effect of drip rate was determined on both the hydrogenated soybean oil and polyethylene glycol 4000 at temperatures of 55° and 70° using an orifice diameter of 3.5 mm. and 5.5 mm. Drop size was measured at flow rates of 15, 30, 60, 90, 120 drops per minute. The surface level was maintained at 10 cm. The data are presented in Fig. 7.

The effect of variations in surface level was also determined. Unless the apparatus is properly designed the liquid level would normally diminish as the liquid drops out through the orifice. This could produce a gradual change in pill weight. The drop weight was determined at constant surface levels maintained at 5, 10, 15, and 20 cm. above the orifice. This procedure was utilized at temperatures of 55° and 70° and at drip rates of 60 and 100 drops per minute for the soybean oil and at drip rates of 60, 90, and 120 drops per minute for the polyethylene glycol 4000. The results are shown in Fig. 8.

As an example of the production of an actual medicated pill the following formula was prepared as the melt.

Phenobarbital sodium	20 Gm.
Glycerin	10 Gm.
Polyethylene glycol 4000	60 Gm.
(To make 1,000 pills)	90 Gm.

The phenobarbital sodium was not soluble in polyethylene glycol so it was first dissolved in the glycerin and then incorporated into melted polyethylene glycol 4000. A drop weight of 90 mg. was obtained using a 6-mm. orifice, a temperature of 60°, and a drip rate of 30 drops per minute. Corn oil was used as the cooling liquid and the pills were washed with petroleum ether to remove traces of corn oil. Each of 100 pills was weighed on an analytical balance to determine the uniformity of drop weight. The data are portrayed graphically in Fig. 9 which illustrates the unusually limited variation in dosage obtained.

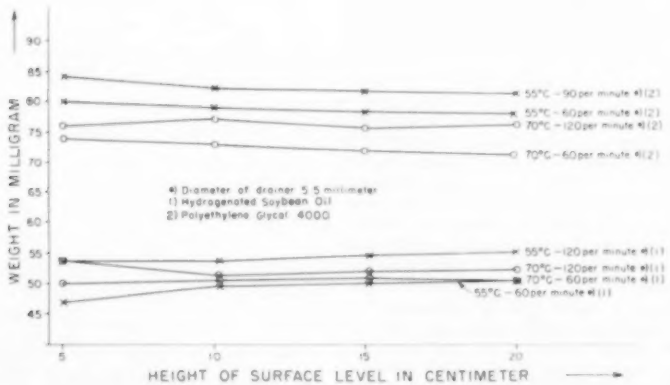
WEIGHT IN MILLIGRAM
HEIGHT OF SURFACE LEVEL IN CENTIMETER
55°C – 90 per minute #(2)
55°C – 60 per minute #(2)
70°C – 120 per minute #(2)
70°C – 60 per minute #(2)
#) Diameter of drainer 5.5 millimeter
1) Hydrogenated Soybean Oil
2) Polyethylene Glycol 4000
55°C – 120 per minute #(1)
70°C – 120 per minute #(1)
70°C – 60 per minute #(1)
55°C – 60 per minute #(1)

Fig. 8.—Influence of the height of surface level on the weight of the pills produced by the Drop Method.

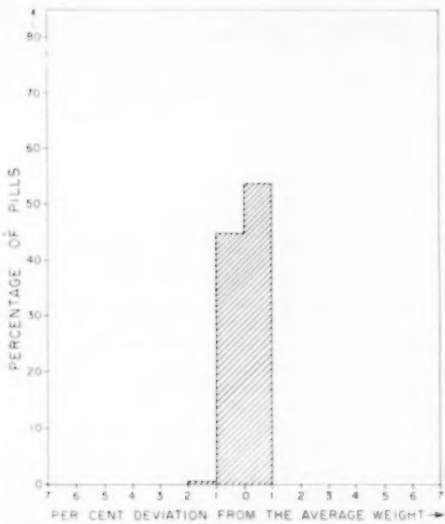

Fig. 9.—Variation in weight of phenobarbital sodium pills processed by the Drop Method. (Experimental—Bjornsson, S.) 1,800 per hour. Av. wt., 90.15 mg.

DISCUSSION

The data presented demonstrate that pills can be produced by the drop method using either water-insoluble or soluble base material and that these pills vary but slightly in weight if factors such as temperature, drip rate, and liquid level are maintained at constant or near constant levels. Variation in weight is considerably less than that to be expected in dosage form such as tablets and capsules.

The small size of the pills formed by this process limits its application to substances with low dosage, probably not exceeding 30 to 50 mg. unless the material itself can be formed into pills by the drop method.

When applicable this method should offer unusual opportunities for controlling disintegration characteristics of tablets. Coloring can be accomplished by adding color to the melt. This method of preparing accurate dosage forms warrants further study.

SUMMARY

1. Preparation of pills by the drop method, previously limited to fatty materials, has been shown to be applicable to water-soluble or dispersible material.

2. A quantitative determination of the effect on pill weight by variations in dropping orifice dimensions, temperature of the melt, drip rate, and liquid level was made.

3. A water-soluble sodium phenobarbital pill was prepared and the variation in individual pill weight determined. Variation in weight did not exceed 2 per cent.

4. The possibilities for control of disintegration time and other characteristics by this method warrant further study.

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A Study of a Radiometric Analysis of Phenobarbital*

By MYRON PAIKOFF and JOHN E. CHRISTIAN

A procedure employing mercury-203 has been developed which is applicable to barbiturate analyses of levels as low as 12.5 micrograms with an overall error of less than 7 per cent. This procedure is capable of analyzing barbiturate levels as low as 1.0 micrograms with an overall error of less than 25 per cent. Barbiturate samples to be analyzed must be free of impurities which react to form insoluble substances with the mercury.

REVIEWS on the analytical applications of radioactive isotopes indicated that the majority of labeling reagents used for analyzing the various medicinal products have been organic in nature (1, 2). However, the literature appears devoid of reports utilizing inorganic salts as labeling reagents for the various medicinal products, although these salts have been successfully used as reagents for "spot tests" for many of these materials.

The work reported here consisted of studying

* Received April 13, 1956 from the Bio-Nucleonics Laboratory, School of Pharmacy, Purdue University, Lafayette, Ind.
Presented to the Scientific Section of the A. Ph. A., Detroit meeting, April, 1956.

the possibilities of employing inorganic salts in a radiometric analysis of an organic compound in microgram quantities. Phenobarbital, a typical medicinal substance, was chosen for the study of the possibilities.

EXPERIMENTAL

Preliminary Studies.—Cobalt, silver, and mercury salts were studied as possible labeling reagents, since they all possessed suitable radioactive isotopes and were capable of forming a reaction product with phenobarbital (3–5). Mercury, in the form of the perchlorate was found to offer the best possibilities and was chosen for further studies and development of the method.

Preparation of the Labeling Reagent.—Mercury-203[1] was selected as the labeling isotope. It was obtained as a nitrate solution having a specific activity of 187 mc./Gm. Exactly 14.0 mg. of mercuric nitrate was added to a 10-lambda aliquot of the mercuric-203 nitrate solution, corresponding to approximately 0.2 μc. The nitrate, contained in a crucible, was decomposed to the oxide by the application of heat from a Bunsen burner. The oxide was then converted to the perchlorate by the addition of approximately 15 lambda of a 60% perchloric acid solution. The mercuric perchlorate was washed into a test tube, previously calibrated to 1.0 ml., with distilled water. Sufficient distilled water was then added to bring to volume. The resulting reagent solution contained approximately 180 μg. of mercuric-203 perchlorate with an approximate radioactivity of 2 μc. in each 10-lambda aliquot. The chemical conversion of mercuric nitrate to mercuric perchlorate is essentially 100%.

Preparation of the Radioactive Derivative.—Strips of Whatman No. 1 filter paper 5 × 42 cm. were used as the "test tube" for the preparation of the radioactive derivative. The paper was spotted 10 cm. from the top with an excess (10 to 20 lambda depending on the level of the analysis) of the mercuric-203 perchlorate reagent. The phenobarbital ranging from 1.0 to 200 μg. was always added to the chromatogram in a 50 lambda aqueous solution. These volumes were maintained to keep the reaction area within approximately a 2.5-cm. diameter.

The paper was placed in a chromatography jar and a front of distilled water was allowed to pass over the reaction area for forty-eight hours. The aqueous front removed the excess labeling reagent carrying it down the strip; whereas the mercury derivative of the phenobarbital being water insoluble remained at the origin.

To determine the feasibility of the method, a series of 4 chromatograms was prepared, each containing a 20-lambda aliquot of the mercuric perchlorate reagent and an excess of phenobarbital (approximately 750 μg.). After the forty-eight hour washing period, the chromatograms were removed from the chromatography jar, dried, and cut into 10 equal segments. The radioactivity of each of the segments was determined using a Geiger-Müller tube. The third segment, the site of the reaction, contained the major portion of the activity. The corrected activities of the radioactive derivative in each of the 4 chromatograms were within a 2% error. A tailing effect of less than 10% did occur in the fourth segment and may be attributed to radioactive species other than mercury in the original sample and/or to a minor solubilization of the radioactive derivative.

Radiometric Analysis of Phenobarbital.—The radioactivity of the formed derivative was determined in two ways. Following the prescribed purification procedure described above, the segment of the chromatogram containing the insoluble radioactive derivative was cut out and placed directly under a Geiger-Müller tube[2] and the activity determined. This method of determining the radioactivity of the derivative is referred to as the "Direct Method." The segment was then placed in a 10-cm. diameter watch glass and 10 mg. of carrier, unlabeled mercuric phenobarbital dissolved in 3 ml. of approximately 1.0 normal sodium hydroxide solution was added. Additional sodium hydroxide solution (approximately 10 ml.) was added to completely saturate the segment containing the radioderivative. Following an elution period of one hour, the segment of the chromatogram was washed free of the solubilized derivative using distilled water. The eluted derivative solution was centrifuged to remove solid impurities, precipitated by the dropwise addition of glacial acetic acid, and localized by centrifugation. The derivative was then washed with four successive portions of distilled water to remove soluble impurities, deposited uniformly on a tared counting cup, dried, weighed, and the radioactivity determined. The corrected specific activity was calculated and expressed in terms of c.p.m./mg.

The levels of radioactivity of the formed derivative as determined by the "Direct Method" and by the "Carrier Method" were separately plotted against micrograms of phenobarbital. Each point on the plot was an average value for six determinations. In each instance, the relationship between concentration and activity made possible the determination of unknown barbiturate quantities by reference to the plot. For careful quantitative work, several points should be established on either side of the unknown prior to calculating the concentration.

After development to satisfactory requirements, the method was applied to a series of barbiturate concentrations ranging from 1.0 to 200 μg. using reagent solutions of varying specific activity. The specific activity was increased by lowering the amount of unlabeled mercuric nitrate added in the preparation of the reagent. A typical set of data is presented in Table I. Similar results were obtained using reagent solutions of different specific activities. Levels as low as 12.5 μg. were analyzed with errors of not more than 7% in each instance, and levels as low as 1.0 μg. were analyzed with errors of not more than 25%; however, errors as low as 3% were obtained in several instances at this lower level (Table I).

[1] The isotope used in this research was granted on allocation from the United States Atomic Energy Commission.

[2] For counting purposes, a Radiation Counter tube having a thickness of 2.8 mg./cm.², connected to a Tracerlab 64 Scaler, Model SC-2A, was used. All samples were counted of sufficient duration to give an overall error of not more than 1.0%. Background, resolution, and decay corrections were made whenever necessary.

TABLE I.—RESULTS FROM THE RADIOMETRIC ANALYSIS OF PHENOBARBITAL LEVELS BETWEEN 100 AND 1.0 MICROGRAMS

Weight of Phenobarb., μg.	Carrier Method[a] Av. Corr. Sp. Act., c. p. m./mg.	Carrier Method[a] Av. Recovery, %[c,d]	Direct Method[b] Av. Corr. Act. (c. p. m.)	Direct Method[b] Av. Recovery, %[c,d]
100	3601[e] ± 72[f]	101	11353 ± 365[e]	99
50	1692 ± 71	95	5740 ± 213	101
25	909 ± 19	100	3215 ± 68	102
12.5	465 ± 27	95	1671 ± 91	100
1.0	62 ± 4	95	266 + 19	97

[a] Method consists of eluting the purified radioactive derivative from the chromatogram, adding 10 mg. of carrier, and determining its specific activity.

[b] Method consists of directly determining the radioactivity of that segment of the chromatogram containing the radioactive derivative.

[c] Based upon the average of six separate determinations calculated from the activity-microgram relationship previously determined with reagent of the same specific activity, using known weights of phenobarbital.

[d] The 200-microgram level of phenobarbital was analyzed in a different series employing labeling reagent of lower specific activity. An average per cent recovery of 101 by the "Carrier Method" and 98% by the "Direct Method" was obtained as described above.

[e] Averages of six separate determinations.

[f] Standard deviation of the mean of six separate determinations was calculated from the formula: $M = \sqrt{\Sigma\, d^2/n(n-1)}$

Determination of Phenobarbital in Serum.—In order to study the practicability of this procedure, samples of serum containing known levels of phenobarbital were analyzed for their barbiturate content. The extraction procedure used in the analysis is essentially the one described by Goldbaum (6). Equine serum was used in all analyses.[2]

Four 10-ml. samples of serum in concentrations of 1.0 μg. of phenobarbital per ml. were analyzed according to the previously mentioned procedure. Average recoveries of approximately 174% by the "Carrier Method" and 165% by the "Direct Method" indicated that impurities also capable of reacting with mercuric-203 perchlorate were being extracted along with the phenobarbital in the chloroform.

A more elaborate procedure and the use of paper partition chromatography to purify the original chloroform extract failed to quantitatively isolate the phenobarbital in a state of purity necessary for analysis according to the previously described radiometric procedure.

DISCUSSION

The two modifications of the procedure, the "Carrier Method" and the "Direct Method," gave similar results as to sensitivity, precision, and accuracy. Therefore, the "Direct Method," being more rapid and simple, is the method of choice.

The various isotope dilution procedures of analysis where they can be applied, offer the advantage, among others, that quantitative separation of the desired constituent is not necessary. It is only necessary to isolate a sufficient quantity of the material being analyzed in a pure state for specific activity determinations. In the radiometric method developed by the work reported here it is not possible to separate the radioactive derivative from other impurities; therefore, it is necessary to quantitatively separate the phenobarbital from impurities which will react to form an insoluble mercury derivative. This puts a limitation on this procedure for the analysis of barbiturates in complex mixtures like serum. Only if the barbiturate can be extracted quantitatively from interfering substances can this procedure be utilized. This is a difficult task especially at microgram and submicrogram levels.

This method, however, where it can be applied shows the highest degree of sensitivity with satisfactory precision of any method yet reported and could be used also for the analysis of other barbiturates. Pedley (5), using the same reagent in a volumetric analysis at the macro level, successfully analyzed other barbiturates.

Although only levels as low as one microgram were analyzed, this procedure theoretically should be sensitive to submicrogram levels if a labeling reagent of sufficiently high specific activity is available.

[2] Obtained from Globe Laboratories, Fort Worth, Tex.

SUMMARY

1. A procedure of analyzing phenobarbital was developed utilizing mercury-203 as the labeling isotope.

2. The above-mentioned analytical procedure was found capable of analyzing levels of phenobarbital as low as 1.0 microgram. Both modifications, the "Carrier Method" and the "Direct Method," gave relatively the same degree of accuracy and precision and should be applicable to the analysis of other barbiturates.

3. The method is limited to the analysis of barbiturate samples free of impurities which react with mercury to form insoluble substances.

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The Effect of Certain Antispasmodics on the Oxygen Uptake of Rat Jejunum and Liver*

By S. T. COKER† and T. S. MIYA

The direct manometric method of Warburg was employed for an *in vitro* study of antispasmodics. Ringer-phosphate buffer (*p*H 7.4) with 0.2 per cent glucose was used as the respiration medium. Papaverine was found to be the most potent inhibitor of tissue respiration using rat jejunum slices, and it had little effect on jejunum homogenates. Varying degrees of inhibition were noted with other antispasmodics tested. It was not possible to correlate antispasmodic activity with inhibition of oxygen uptake. Acetylcholine, eserine, and methylene blue did not antagonize the inhibition caused by the antispasmodics. Acetylcholine increased the oxygen uptake of control liver with little or no effect on the jejunum slices. Qualitatively, the antispasmodics produced the same effects on the liver as on the jejunum slices.

VARIOUS ANTISPASMODICS have been found to inhibit the oxygen uptake of tissues other than smooth muscle (1–5). Schmitt and Nicoll (6) noted that the cytochrome system was necessary for maintaining muscle tone. Cyanide in very low concentrations was able to inhibit reversibly the stimulating effect of acetylcholine, pilocarpine, and barium chloride on smooth muscle.

In view of these reports the effects of certain antispasmodics on the oxygen uptake of smooth muscle and liver have been studied.

EXPERIMENTAL

Preparation of Animals and Tissues.—Nonfasted adult male animals were used throughout this investigation except where indicated. Sprague-Dawley rats were fed on a diet of Purina Laboratory Chow and the guinea pigs were fed on Purina Rabbit Chow Checkers supplemented with vitamin C in their drinking water. The animals were killed by a blow on the head. The tissues were removed and placed in ice-cold Ringer-phosphate buffer solution *p*H 7.4 with 0.2% glucose. The liver slices were prepared with a Stadie-Riggs microtome (7). Various techniques have been used for studying the oxygen uptake of smooth muscle (8–14). The jejunum slices, free of mucosa, were prepared by modifying the technique of Dickens (10). Ten per cent jejunum homogenates were prepared in Ringer-phosphate buffer solution using a 25 × 200 mm. Potter-Elvehjem glass homogenizer. Suspension of slices and homogenization of tissues were carried out in ice-cold Ringer-phosphate solution with 0.2% glucose. Trial experiments confirmed the effect of dilution of homogenates on O_2 uptake (15).

Addition of Drugs.—The drugs were dissolved in Ringer's solution. Addition to the buffer medium resulted in the formation of a milky suspension of the least soluble drugs. Trial experiments indicated that the drugs could suitably be added to the tissue just before placing the flask on the manometer rather than tipping after thirty minutes. Quantitatively the results were the same. A similar procedure was employed by Smith, *et al.* (16).

Manometric Procedure.—Employing the direct method of Warburg, the rate of O_2 uptake was determined on a wet weight basis and expressed as microliters of O_2/100 mg. of tissue/2 hours or as specified. The slices of tissue were weighed on a microtorsion balance. The gas phase was air. The center well of the flasks contained 0.2 ml. of 10% KOH with a cylinder of Whatman no. 40 filter paper to aid in the absorption of CO_2. The rim of the center well was coated with hydrous lanolin. The water bath temperature was 38°. The equilibration period was ten minutes for the homogenates and fifteen minutes for the slices. Readings were made every twenty minutes for the slices and thirty minutes for the homogenates. The shaking rate was 116/minute and the stroke was 3 cm. All drug concentrations refer to the final flask concentration.

RESULTS

The Effect of Antispasmodics on the Oxygen Uptake of Rat Jejunum Homogenates.—The antispasmodics were compared on the basis of the molar concentration required to produce a 50% inhibition of O_2 uptake. Atropine, Benadryl®, papaverine, Probanthine®, Pamine®, Pavatrine®, and sodium nitrite were screened, but only atropine, Probanthine®, and Benadryl® were found to be capable of producing at least 50% inhibition of O_2 uptake. Three dose levels, bridging the approximate inhibition^{50} dose with a ratio between doses of 1.5 and a total of 3 flasks per dose of drug, were plotted on a DeBeer's nomograph (17). The three drugs were studied simultaneously in 7 consecutive experiments and the mean inhibition^{50} doses analyzed by the analysis of variance and the Duncan technique (18). This analysis allows the drugs to be tested

* Received April 13, 1956, from the Purdue University School of Pharmacy, Lafayette, Ind.
Presented to the Scientific Section, A.PH.A., Detroit meeting, April 1956.
The authors are indebted to Searle and Company for the Probanthine® (β-diisopropylaminoethylxanthene-9-carboxylate) and Pavatrine® (β-diethyl-aminoethyl fluorene-9-carboxylate); Ciba Pharmaceutical Products, Inc., for the Trasentine® (diphenylacetyldiethylaminoethanol); The Upjohn Company for the Pamine® (scopolamine methylbromide) and Parke, Davis and Company for the Benadryl® (β-dimethylamino ethyl benzhydryl ether).
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for significant difference and ranked. It was assumed that there was a homogeneous deviation for each 50% point. The antispasmodics were not studied extensively using 10% jejunum homogenates, since the O_2 uptake was very low and the tissue slices offered a much better medium for studying the inhibition. The results appear in Table I.

verine. The molecular weight of the base was used in calculating the doses. No significant difference in O_2 uptake of jejunum "slices" was noted between the treated and control animals.

Antagonism of the Inhibition of Oxygen Uptake Caused by Certain Antispasmodics.—Jejunum as well as liver slices were used in this study. An at-

TABLE I.—THE INHIBITION[a] DOSES OF CERTAIN ANTISPASMODICS ON RESPIRATION OF RAT JEJUNUM HOMOGENATES AND RAT AND GUINEA PIG SLICES

Drug	Type of Tissue	Prep.	Species of Animal	$\bar{X}^a$	Standard Deviation
Atropine[b]	Jej.	Homog.	Rat	1.34×10^{-1}	0.12
"	"	Slices	"	3.10×10^{-2}	0.53
"	"	"	Guinea Pig	1.76×10^{-2}	0.17
Probanthine®	"	Homog.	Rat	7.00×10^{-2}	0.86
"	"	Slices	"	5.20×10^{-3}	0.13
"	"	"	Guinea Pig	3.13×10^{-3}	0.56
Benadryl®	"	Homog.	Rat	8.30×10^{-2}	1.12
"	"	Slices	"	3.90×10^{-3}	0.82
"	"	"	Guinea Pig	3.02×10^{-3}	0.28
Trasentine®	"	"	Rat	2.70×10^{-3}	0.27
Papaverine[c]	"	"	"	7.85×10^{-5}	1.06

[a] Each $\bar{X}$ is the molar concentration required to produce 50% inhibition of O_2 uptake in 7 experiments (21 flasks).
[b] Atropine was significantly less effective than the other antispasmodics ranked above at 1% level.
[c] Papaverine is obviously the most effective inhibitor among the drugs studied on jejunum slices.

The Effect of Antispasmodics on the Oxygen Uptake of Rat and Guinea Pig Jejunum Slices.—Atropine, Probanthine®, Benadryl®, Trasentine®, and papaverine were found to be capable of producing at least 50% inhibition of O_2 uptake. The inhibition[50] doses were determined and the data analyzed as previously described. Atropine, Benadryl®, and Probanthine® were studied simultaneously on both rat and guinea pig jejunum slices. The inhibition[50] for Trasentine® and papaverine was determined only on rat jejunum slices. The guinea pig was used only in a limited number of experiments in an effort to detect species variation in the response to the antispasmodics. The drugs are ranked in Table I.

Determination of Normal QO_2 for Guinea Pig and Rat Jejunum Slices.—Since little information is available concerning the normal QO_2 for guinea pig and rat jejunum slices, it was determined in the following manner. After the tissue had been placed in the flasks and the experiment was under way, two additional samples of tissue were blotted, weighed, and placed on a tared piece of aluminum foil to be dried in an oven at 100° for twenty-four hours. The dry/wet ratio was determined and used to calculate the weight of dry tissue that would be present in a weighed sample of tissue in the control flasks. The QO_2 is defined as the μL. of O_2 uptake/mg. dry weight/hour. The normal QO_2 for the guinea pig jejunum slices was 4.86 ± 0.42. The normal for the rat jejunum slices was 3.23 ± 0.35.

Oxygen Uptake of Jejunum Slices from Rats Treated with Certain Antispasmodics.—The effects of antispasmodics on tissue respiration when the drugs were injected subcutaneously into rats two hours before sacrificing were studied. Pamine® 23 mg./Kg., Probanthine® 26 mg./Kg., atropine 40 mg./Kg., and papaverine 200 mg./Kg. were injected in equal molar concentrations, except papatempt was made to confirm Cavanaugh's (2) partial success in antagonizing atropine on rat brain homogenates using acetylcholine and eserine. In this study acetylcholine, methylene blue, and eserine were unable to antagonize the inhibition produced by the antispasmodics employing jejunum and liver slices. On the rat liver slices, acetylcholine 10^{-2} molar caused an increase in oxygen uptake, but failed to antagonize the inhibition caused by atropine 10^{-2} molar (Fig. 1). Methylene blue in final

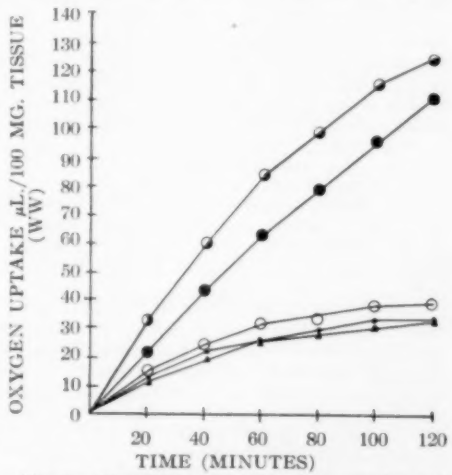

Fig. 1.—The effect of acetylcholine, atropine and scopolamine on rat liver slices. Each point is a mean of 2 flasks. ●—● Control liver; ⊖—⊖ acetylcholine 10^{-2} *M*; ⊖—⊖ atropine 10^{-2} plus acetylcholine 10^{-2} *M*; ●—● atropine 10^{-2} *M*; ▲—▲ scopolamine 5×10^{-2} *M*.

concentrations of 0.005% caused as much as a 34.00% increase in the oxygen uptake of jejunum slices and 24.22% increase in the oxygen uptake of rat liver slices. Although methylene blue produced this increase in the oxygen uptake of control jejunum and liver, it failed to antagonize the inhibition produced by the antispasmodics.

the comparative study, scopolamine and atropine appeared to inhibit jejunum and liver to a similar degree. Scopolamine and Pamine® serve as an additional example of how slight changes in the molecular configuration of a compound can modify its action in metabolic processes.

Benadryl®, Trasentine®, and Probanthine® ap-

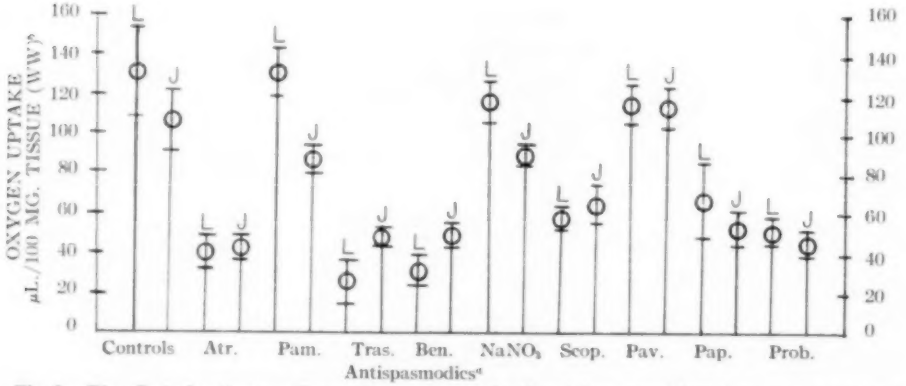

Fig. 2.—The effect of antispasmodics on the oxygen uptake of rat jejunum and liver slices. [a]The antispasmodics were compared in equal molar concentrations. The concentrations were as follows: atropine sulfate, 3×10^{-2} *M*; Pamine bromide, 10^{-2} *M*; Trasentine hydrochloride, 3×10^{-2} *M*; Benadryl hydrochloride, 4×10^{-2} *M*; $NaNO_2$, 10^{-2} *M*; scopolamine hydrobromide, 3×10^{-2} *M*; Pavatrine hydrochloride, 3.62×10^{-4} *M*; papaverine hydrochloride, 8×10^{-5} *M*; Probanthine bromide, 5×10^{-2} *M*. [b]The given oxygen uptake is a mean total μL. oxygen uptake with the standard deviation/100 mg. tissue (WW) at two hours/3 experiments.

A Comparative Study of the Antispasmodics on Liver and Jejunum.—Each antispasmodic was studied on the jejunum and liver from the same animal. The mean total μL. of oxygen uptake of three such experiments appear in Fig. 2. Both tissues were prepared as previously described. The doses of the antispasmodics, wherever possible, were the inhibition[10] doses as determined on the rat jejunum slices. It can be seen that the antispasmodics produce equal or greater inhibition of the respiration on liver slices than on jejunum slices.

DISCUSSION

These studies indicate that jejunum slices, which have had the mucosa removed, are thin enough to give consistent oxygen uptake for as long as two hours. This confirms the investigations of Dickens (10).

The dose of atropine, Benadryl®, and Probanthine® necessary to produce a 50% inhibition of oxygen uptake of jejunum homogenates was at least ten times the dose required for jejunum slices. It was also interesting to note that papaverine caused little inhibition of the respiration of homogenates, while it caused the greatest inhibition in jejunum and liver slices (Fig. 3).

Pamine®, a potent antispasmodic, caused no significant inhibition of oxygen uptake. Scopolamine, a homolog of Pamine®, caused a significant inhibition of liver and jejunum. This agrees favorably with the findings of James (1) in his study of the effect of scopolamine on placental tissue. In

pear to inhibit rat jejunum slices to the same degree as indicated by similar inhibition[10] doses. The diphenyl group, which is contained in Trasentine® and Benadryl®, is said to be responsible for the inhibi-

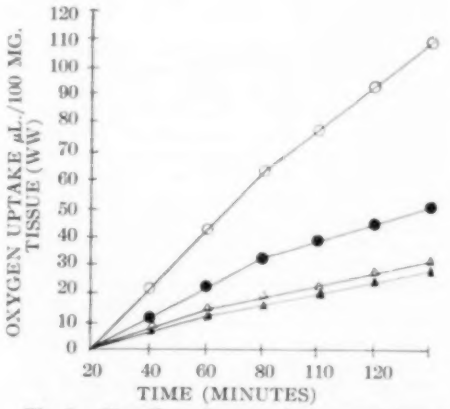

Fig. 3.—The effect of papaverine on rat jejunum slices and homogenates. Each point is a mean of 3 flasks.
⊖——⊖ Control jejunum slices; ●——● jejunum slices with papaverine 6.66×10^{-5} *M*; △—△ control 10% jejunum homogenates; ▲—▲ ten per cent jejunum homogenates with papaverine 10^{-2} *M*.

tion of the cytochrome oxidase system (4, 5). Schmitt and Nicoll (6) found that the cytochrome system was involved in maintaining tone in smooth muscle. The inhibition of this system might conceivably be the mechanism of action for some antispasmodics.

Papaverine produced a 50% inhibition of oxygen uptake of jejunum slices at a significantly lower dose than any of the antispasmodics tested. It is known that papaverine is primarily a musculotropic antispasmodic. The barbiturates have an antispasmodic action *in vitro* and they also inhibit tissue respiration (19, 20).

There appeared to be no significant inhibition of oxygen uptake in the tissue taken from the treated animals. It is possible that the tissue may have been inhibited while *in vivo*, but the oxidative processes resumed at a normal level when the tissue was removed from the treated animal and placed in a flask containing no drug. Atropine has been shown to depress the oxygen uptake of the brain *in vivo* (21).

Methylene blue 0.005% increased the oxygen uptake of both jejunum and liver. Acetylcholine 10^{-2} molar increased the liver but not the jejunum oxygen uptake. This difference in effect may be due to the absence of choline oxidase in the jejunum (22).

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Composition of Gum Turpentines of Pines XXVIII.*

A Report on *Pinus edulis* from eastern Arizona, *P. tropicalis* from Cuba, and *P. elliottii* var. *densa* from Florida

By N. T. MIROV and P. M. ILOFF, Jr.

Composition of gum turpentine of three pine species is reported. The turpentine of *P. edulis* contained: *d* and *dl*-α-pinene, 46%; unidentified terpenes, 1%; myrcene, 2%; *d*-Δ3-carene, 6%; ocimene 1%; *dl*-limonene, 1%; terpinolene 1%; ethyl caprylate, 6%; unidentified mixture of oxygenated compounds and hydrocarbons, 12%; sequiterpenes related to cadinene, 16%; residue and loss, 8%. The turpentine of *P. tropicalis* consisted of: *d* and *dl*-α-pinene, 96%; *d* and *dl*-limonene, 1–2%; an unidentified terpene, 1%, and pot residue and loss 1.6%. The turpentine from *P. elliottii* var. *densa* contained: *l*- and *dl*-α-pinene, 71%; *l*-β-pinene, 3–4%; unidentified terpene, 1–2%; *l*-β-phellandrene, 19%; methyl chavicol, 3%; pot residue and loss 1.2%.

PINUS EDULIS

THE BOTANICAL NAME of *Pinus edulis* Engelm. means in English an edible pine. The pine's large seeds are used for food. Its common name, pinyon, is Spanish. It grows chiefly in Utah, Colorado, Arizona, and New Mexico, but extends to the trans-Pecos region of Texas, to the Mexican province of Chihuahua, and possibly to the northern part of Lower California. Apparently it crosses with the other three pinyon pines: *Pinus monophylla* of Nevada, *P. quadrifolia* of southern California, and *P. cembroides* of Mexico. *P. edulis* turpentine obtained in southwestern Colorado (southeast of Mancos) was analyzed by Schorger (10) and reported to have the following composition: *d*-α-pinene, 70 to 75%; *l*-β-pinene, 5%; and *d*-cadinene, 15 to 20%.

* Received January 9, 1956, from the Institute of Forest Genetics, California Forest and Range Experiment Station, maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.
The work reported in this paper was aided through a grant from the Rockefeller Foundation.

Oleoresin samples of *Pinus edulis* used in this study were obtained from the Fort Defiance plateau (elevation 6,800 to 7,400 feet), Navajo Indian Reservation, through the courtesy of Mr. Horace S. Haskell. The oleoresin was distilled under reduced pressure, so that at the end of distillation, the temperature reached 203° and the vacuum gage registered 4 mm. pressure of mercury. Yield of turpentine was 24% of the weight of the oleoresin.

The turpentine had the following characteristics: d_4^{22}, 0.8708; n_D^{22}, 1.4728; $[\alpha]_D^{22}$, +17.9. A charge of 1,083 Gm. of the turpentine was distilled through a 90-cm. long, 10-mm. inside diameter Vigreux column equipped with a dually-heated Todd jacket. A reflux ratio of 2:1 was used. The results of this preliminary distillation are shown in Table I. Fraction A was distilled by use of a 90-cm. long, 25-mm. inside diameter Todd column packed with glass helices. After 24.0% of the original 1,083 Gm. was received, fraction B was added to the pot. Fraction C and the pot residue were added after reaching head temperature of 45 and 71°, respectively. An automatic head[1] was used to maintain a reflux ratio of 10:1. The results of the distillation are shown in Table II.

TABLE I.—PRELIMINARY DISTILLATION OF GUM TURPENTINE OF PINUS EDULIS

Fraction	Boiling Range, °C.	Pressure, mm.	Distillate, %
A	43–48	14	47.5
B	48–56	14	8.4
C	56–108	14	13.6
Pot residue	...	...	29.2
Loss	...	...	1.3

The physical properties of fractions 1 and 2 were close to those of α-pinene. From 7 ml. of fraction 1, 0.4 Gm. of *dl*-α-pinene nitrosochloride, melting at 102–103°, was prepared. After several recrystallizations, the derivative melted at 104–105° and showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrosochloride.

The negative rotation of fractions 8 and 9 suggested the possible presence of β-pinene. A 5-Gm. sample of fractions 8 and 9 was oxidized with alkaline permanganate but nopinic acid was not isolated.

The low density of fraction 10 suggested the presence of β-myrcene. The fraction gave a very cloudy solution in alcohol so the U. V. absorption was not determined. Five Gm. of fraction 10 was heated to 100° for ten minutes with 1 Gm. of maleic anhydride. The mixture was cooled and dissolved in petroleum ether (b. p. 30–60°) and then allowed to stand at −5° for a week. The crude adduct (0.62 Gm.) was filtered off and recrystallized several times from hexane at −5°. The purified adduct melted at 33–34° and its melting point was not depressed on admixture with authentic maleic anhydride adduct of β-myrcene.

The higher densities, positive optical rotations, and sweet odor of fractions 11 to 14 indicated the possible presence of Δ^3-carene. Five grams of fraction 12 was used to prepare 0.80 Gm. of crude nitrosate which, after two recrystallizations from chloroform by addition of cold methanol, melted at 144–145°. The melting point was not depressed on admixture with authentic Δ^3-carene nitrosate.

The densities of fractions 15, 16, and 17 decreased to a minimum at fraction 17. The odor of fraction 17 was similar to the odor of ocimene-containing fractions of the turpentine from *P. quadrifolia* (9). The U. V. absorption of fraction 17 was λ max. 235 mμ; ε max. 10,400 in 95% ethanol.[2] The ultraviolet absorption of ocimene is reported in the literature (15) as λ max. 237 mμ; ε max. 16,400 in iso-octane. Excepting for the lower intensity of fraction 17, the curve was very similar to the one recorded in the literature. A sample of fraction 17 was reduced with sodium in alcohol. The resulting hydrocarbons were isolated, taken up in ether, and bromine was added drop by drop, keeping the temperature near 0°. After ether was removed an oil was obtained from which a small amount of *dl*-limonene tetrabromide was isolated by crystallization from methanol at −5°. From the mother liquor of this crystallization, crystals were obtained which, after three recrystallizations from chloroform by addition of methanol, melted at 93.5–94.5°. Dihydro-ocimene tetrabromide (dihydromyrcene tetrabromide) was reported to melt at 95–96° (1). One milliliter of fraction 17 which was allowed to stand in an open beaker for three days became very viscous and syrup-like. Ocimene (12) is known to behave in a similar manner. It is concluded fractions 15 to 19 contained ocimene and *l*- and *dl*-limonene.

Fraction 20 had physical properties near the properties of terpinolene (13). A 2-ml. portion was treated with bromine using the method of Berry (2). The tetrabromide was recrystallized several times from ethyl acetate until it melted at 117–118°. Melting point of the tetrabromide showed no depression when mixed with authentic terpinolene tetrabromide.

Fractions 21 and 22 were transition fractions containing both hydrocarbons and oxygenated compounds.

Fractions 23 and 24 boiled over a narrow range and had a sweet odor which is probably responsible for the pleasant odor of the oleoresin itself. The physical properties of these fractions indicated the possible presence of an aliphatic ester.

One gram of fraction 24 was treated with benzylamine according to the procedure of Dermer and King (7). The amide (428 mg., m. p. 62–63.5°) was recrystallized from diluted ethanol to a constant melting point at 65–66°. By the same procedure, a 61% yield of the crude benzyl amide of caprylic acid, m. p. 63–64.5°, was prepared from caprylic acid. The amide (white crystals) was recrystallized to a constant melting point of 65.5–66.5°.

Anal.—Calcd. for $C_{15}H_{23}ON$: C, 77.20%; H, 9.94%. Found: C, 77.06%; H, 9.28%.

This derivative was not previously reported. A mixture of the authentic benzyl amide of caprylic acid with the derivative from fraction 24 melted at 65.2–66°.

[1] Manufactured by Todd Scientific Co., Philadelphia, Pa.

[2] All U. V. Absorption measurements reported in this paper were performed on the Beckmann DU model spectrophotometer.

Two milliliters of fraction 24 was treated according to the method of Renfrow and Chaney (14), with 2 drops of concentrated sulfuric acid and 1.5 Gm. of 3,5-dinitrobenzoic acid. After one recrystallization of the crude product from ethanol, 431 mg. of needles, m. p. 89–93°, was obtained. After 2 more crystallizations, 203 mg. of derivative, melting at 92.5–93.5° was obtained. When mixed with authentic ethyl 3,5-dinitrobenzoate, the derivative showed no depression of melting point. Thus the acid portion of the ester was caprylic acid and the alcohol portion ethanol. Therefore, the ester was ethyl caprylate.

A series of oxygenated fractions with positive optical rotations reaching a maximum rotation at fraction 39 distilled over after the ethyl caprylate. Attempts to prepare a *p*-nitrobenzoate and 2,4-dinitrophenylhydrazone failed. An analysis showed C, 85.28%; H, 11.48%. This indicates that the fraction is mainly hydrocarbon with a sizeable amount of oxygenated material as impurity.

The remainder of the oil consists of sesquiter-

TABLE II.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS EDULIS, P. TROPICALIS AND P. ELLIOTTII VAR. DENSA

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate	Density	Index of Refraction	Specific Rotation
	Pinus edulis (1,083 Gm. used)			d_4^{23}	n_D^{23}	$[\alpha]_D^{23}$
1	13	43–43	23.97	0.854	1.4643	+46.8
2	13	43–44	19.54	0.854	1.4646	+46.9
3	13	44–49	3.29	0.853	1.4653	+30.4
to				to	to	to
6				0.851	1.4669	+2.8
7	13	49–51	0.77	0.846	1.4678	−10.4
8	13	51–53	0.43	0.843	1.4687	−15.9
9	13	53–54	0.39	0.841	1.4692	−16.4
10	13	54–55	0.87	0.837	1.4699	−8.3
11	13	55–56	1.52	0.839	1.4703	+2.6
12	13	56–57	4.66	0.843	1.4708	+10.7
13	13	57–58	0.86	0.848	1.4720	+9.3
14	13	58–59	0.66	0.845	1.4731	+3.6
15	13	59–61	0.42	0.839	1.4750	−1.9
16	13	61–63	0.40	0.831	1.4771	−6.8
17	13	63–66	0.67	0.825	1.4798	−7.0
18	13	66–68	0.42	0.830	1.4815	−3.1
19	13	68–70	0.67	0.843	1.4829	−1.0
20	13	70–71	0.40	0.857	1.4840	+0.7
21	13	71–86	1.07	0.861	1.4447	0.0
22	13	86–88	0.46	0.870	1.4271	0.0
23	13	88–89	1.03	..	1.4208	+0.7
24	13	89–90	3.80	0.866	1.4190	+0.7
25	13	90–93	0.74	0.866	1.4196	+0.9
26	13	93–97	0.38	0.881	1.4291	+0.4
27	13	98–101	0.29	0.894	1.4410	−0.2
28	13	101–103	0.30	0.899	1.4490	0.0
29	13	103–106	0.29	0.900	1.4532	0.0
30	13	106–108	0.32	0.900	1.4574	+1.4
31	13	108–110	0.48	0.900	1.4624	+4.0
32	13	110–111	0.27	0.900	1.4659	+4.0
33	13	111–112	0.47	0.901	1.4687	+5.6
34	13	112–114	1.13	0.903	1.4722	+7.8
35	13	114–115	0.72	0.905	1.4760	+10.6
36	13	115–116	1.00	0.907	1.4797	+11.7
37	13	116–116	1.64	0.906	1.4808	+11.7
38	13	116–117	1.00	0.909	1.4837	+14.1
39	13	117–118	0.99	0.909	1.4866	+15.0
40	13	118–119	0.66	0.911	1.4892	+13.9
41	13	119–120	0.93	0.911	1.4920	+8.6
42	13	120–121	1.27	0.911	1.4935	+2.2
43	13	121–122	1.11	0.913	1.4947	+7.7
44	13	122–124	0.53	0.911	1.4982	−5.1
45	13	124–126	0.67	0.907	1.5008	−23.6
46	13	126–127	0.74	0.903	1.5021	−39.0
47	13	127–129	1.11	0.902	1.5031	−49.2
48	13	129–130	2.20	0.902	1.5042	−56.6
49	13	130–132	2.62	0.903	1.5059	−42.5
50	13	132–133	1.90	0.905	1.5065	−8.5
51	13	133–134	2.39	0.911	1.5069	+20.2
52	13	134–135	1.44	0.912	1.5072	+56.2
53	13	135–136	1.07	0.914	1.5074	+76.8
54	13	...	1.26	0.922	1.5109	+41.5
Pot residue and loss			3.78			

Fractions	Pressure, mm.	Boiling Range, ° C.	Distillate	Density	Index of Refraction	Specific Rotation
	Pinus tropicalis (491 Gm. used)			d_4^{23}	n_D^{23}	$[\alpha]_D^{23}$
1	760	147–155	1.6	0.856	1.4639	+36.6
2	760	155–155.5	85.6	0.853	1.4648	+37.0
3	760	155.5–156.5	7.5	0.853	1.4651	+35.4
4	15	48–49	1.0	0.855	1.4669	+29.5
5	15	49–51	0.5	0.856	1.4688	+23.1
6	15	51–53	0.4	0.855	1.4703	+16.6
7	..	...	1.8	0.842	1.4748	+20.7
Pot residue and loss			1.6			
	Pinus elliottii var. *densa* (684 Gm. used)			d_4^{23}	n_D^{23}	$[\alpha]_D^{23}$
1	18	47.5–48.5	0.4	0.859	1.4630	−45.7
2	18	48.5–49	65.2	0.854	1.4647	−47.2
3	16.5	48–51	2.6	0.855	1.4657	−46.0
4	16.5	51–53	1.7	0.856	1.4681	−39.9
5	16.5	53–54	0.6	0.857	1.4706	−33.9
6	16.5	54–55	1.7	0.858	1.4736	−25.9
7	16.5	55–56	0.3	0.861	1.4745	−22.8
8	17	57–58	0.9	0.858	1.4752	−20.7
9	17	58–60	1.2	0.854	1.4759	−19.3
10	17	60–62	1.2	0.842	1.4775	−22.9
11	17	62–63	2.3	0.833	1.4808	−25.5
12	17	63–64	0.7	0.836	1.4830	−25.4
13	17	64–65	0.3	0.837	1.4846	−22.0
14	17	65–66	3.1	0.838	1.4851	−20.6
15	17	66–67	1.7	0.838	1.4853	−20.0
16	17	67	1.6	0.837	1.4856	−19.4
17	17	67	5.8	0.838	1.4860	−16.8
18	17	67	3.1	0.838	1.4860	−15.4
19	17	67–69	0.8	0.837	1.4856	−14.8
20	17	69–92	0.6	0.842	1.4852	−10.4
21	17	92–100	0.7	0.929	1.5009	−11.0
22	2	...	2.3	0.954	1.5040	−14.9
Pot residue and loss			1.2			

penes, two of which occur in considerable quantity. The fractions in which these occur in highest purity are fractions 48 and 53.

Fraction 48 redistilled at reduced pressure over sodium gave a heart cut with the properties: b_{11}, 126–129°; d_4^{24}, 0.9028; n_D^{24}, 1.5045; $[\alpha]_D^{24}$, −54.1. By treatment of 3 ml. of fraction 48 with dry HCl in ether, 100 mg. of a hydrochloride was isolated which melted at 117–118° and did not depress the melting point of cadinene dihydrochloride. The rotation of fraction 48 hydrochloride in chloroform was $[\alpha]_D^{23}$, −33.7, c = 1.7.

Fraction 53 redistilled at reduced pressure over sodium gave a heart cut with the properties: b_{10}, 133–134°; d_4^{24}, 0.9131; n_D^{24}, 1.5078; $[\alpha]_D^{24}$ +78.4. A 0.74-Gm. sample of a hydrochloride was prepared from 3 ml. of the heart cut which melted at 118–119°; it did not depress the melting point of cadinene dihydrochloride. The optical rotation was $[\alpha]_D^{23}$, −36.8, c = 2.88 in chloroform. The positive rotating fraction 53 gave a hydrochloride of negative rotation as does canadene (11), but the fraction has considerably higher rotation and lower density than canadene.

To summarize, the gum turpentine of *P. edulis* contained: *d*- and *dl*-α-pinene, 46%; unidentified terpenes, 1%; myrcene, 2%; *d*-Δ³-carene, 6%; ocimene 1%; *dl*-limonene, 1%; terpinolene 1%; ethyl caprylate, 6%; unidentified mixture of oxygenated compounds and hydrocarbons, 12%; sesquiterpenes related to cadinene, 16%; residue and loss, 8%.

It is seen from these results that the chemical composition of *Pinus edulis* turpentine is much more complex than reported by Schorger. The exquisite fragrance of the turpentine is to a large extent due to the presence of ethyl caprylate. Ethyl caprylate has never been reported before as a constituent of any essential oil.

PINUS TROPICALIS

Pinus tropicalis Morlet grows at sea level in Cuba where it is associated with *P. caribaea*, and on the nearby island, Isla de Pinos. Shaw places this pine in the group of Lariciones, which consists mainly of Old World species, plus *P. tropicalis* and *P. resinosa* of the American northeast.

A sample of oleoresin was obtained through the courtesy of Sr. Ing. Alberto J. Fors, Director of Forests, Republic of Cuba. The sample was procured at the tract owned by the Minas de Matahambre S. A. under supervision of the company's silviculturist, and was accompanied with specimens of branches and cones.

Turpentine was separated from the oleoresin under reduced pressure. At the end of distillation, pressure was 9 mm. of mercury and temperature of the batch was 200°. Rosin remaining in the distilling flask on cooling became very hard and brittle,

showing that all volatile oil had been expelled. Yield of turpentine was 23.7%.

The crude turpentine had the following physical characteristics: d_4^{21}, 0.8566; n_D^{21}, 1.4658; $[\alpha]_D^{22.5}$ +36.6.

A batch of 491 Gm. of the turpentine was distilled in a 90-cm. long, 25-mm. inside diameter Todd column packed with 3/32-inch single-turn glass helices. Results of the distillation are shown in Table II.

Fraction 2 was collected in 5 parts, approximately 84 Gm. each. These parts had nearly identical properties and were combined. The properties of fraction 2 were close to those of α-pinene. From 7 ml. of fraction 2, 1.1 Gm. of *dl*-α-pinene nitrosochloride, melting at 104–105°, was prepared. The melting point was not depressed by admixture with authentic *dl*-α-pinene nitrosochloride.

Judging from the high density and depression of optical rotation in fractions 10 and 11, they contain a small amount of an unidentified terpene, possibly β-pinene.

Fraction 12 consisted of the tails collected at about 1–3 mm. Fraction 12 was redistilled over sodium and yielded a heart cut (about 1/3) with the following properties: b_{18}, 58–60; d_4^{23}, 0.842; $n_D^{22.5}$, 1.4751; $[\alpha]_D^{23}$, +24.9. From 2 Gm. of the heart cut, 0.33 Gm. of crude tetrabromide was prepared. After three recrystallizations from ethyl acetate (0°), 53 mg. of crystals melting at 124–124.5° was received. Admixture with an authentic sample of *dl*-limonene tetrabromide did not depress the melting point.

To sum up, the gum turpentine of *P. tropicalis* consisted of: *d*- and *dl*-α-pinene, 96%; *d*- and *dl*-limonene, 1–2%; an unidentified terpene, 1%, and pot residue and loss 1.6%.

PINUS ELLIOTTII VAR. *DENSA*

Pinus elliottii var. *densa* Little & Dorman is the slash pine of southern Florida. Formerly, slash pine over its extensive range from Georgia to Honduras was known as *P. caribaea*, but recently this name has been applied only to the slash pine growing in the West Indies and in Central America. Slash pine of the continent is now known as *P. elliottii*. This species, in turn, was subdivided into *P. elliottii* var. *elliottii*—the familiar naval stores tree of our southeast—and *P. elliottii* var. *densa* of southern Florida. There are ample morphological justifications for segregating slash pine into the above three entities. It appeared desirable, therefore, to see if the chemical characteristics of these three pines are different.

Turpentine of *P. elliottii* var. *elliottii* has been studied very well. It consists of about 75% of α-pinene; 21% of *l*-β-pinene and 4% of tails (8). In the tails, Chadwick and Palkin (5) found dipentene, minute amounts of other hydrocarbons and of methyl chavicol, and traces of other oxygenated compounds.

A small sample of turpentine of *Pinus caribaea* (from Nicaragua) was investigated in this laboratory. Its composition was found to be as follows: *l*-α-pinene, 56–60%; *d*-Δ³-carene, 10%; *l*-β-phellandrene, 5%; tails, mostly a sesquiterpene; longifolene, 13–14%; and 11% of polymerized residue (9).

Oleoresin for the present experiment was obtained through the courtesy of the Southeastern Forest and Range Experiment Station. The sample was collected from five trees in Collier County, Florida, about 12 miles south of Immokalee.

A 3,694-Gm. batch of the oleoresin was heated under reduced pressure so that when all turpentine was distilled off, the vacuum gauge showed pressure of 2 mm. of mercury and the temperature reached 190°. Yield of turpentine amounted to 19%.

The turpentine had the following physical characteristics: d_4^{24}, 0.8532; n_D^{25}, 1.4709; $[\alpha]_D^{24}$, −38.6.

A batch of 684 Gm. of the turpentine was distilled through the 90-cm. long, 25-mm. inside-diameter Todd column packed with glass helices. A reflux ratio of 10:1 was maintained to fraction 21. Fraction 22 was received at total takeoff. The results of the distillation are shown in Table II.

Fraction 2 was collected in approximately 100-ml. portions which had nearly identical physical properties. The properties of fraction 2 indicated that it was nearly pure *l*- and *dl*-α-pinene. From 7 ml. of fraction 2, 0.37 Gm. of *dl*-α-pinene nitrosochloride, melting at 104–105°, was prepared. Its melting point was not depressed by admixture with authentic *dl*-α-pinene nitrosochloride.

Fractions 3 and 4 also contained mainly *l*-, and *dl*-α-pinene.

Fractions 5, 6, 7, and 8 had higher densities, which, with the values for refractive index and optical rotation, indicated the probable presence of *l*-β-pinene. A 6.5-ml. portion of fraction 6 was oxidized with alkaline permanganate. The crude nopinic acid (0.12 Gm.) was recrystallized from benzene and then from water. The crystals then melted at 124–125° and the melting point was not depressed by admixture of authentic nopinic acid.

Fractions 9 and 10 were transition fractions.

A plot with the refractive indices of the fractions as abscissas and the densities as ordinates (13) indicated the presence of an unidentified terpene, possibly α-phellandrene, in fraction 11. However, the ultraviolet absorption showed λ max. 231 mμ, ε = 14,100 in 95% ethanol. This indicates the material was highly contaminated with β-phellandrene, which is found in a later fraction.

Fractions 12 to 16 contained increasing amounts of *l*-β-phellandrene.

Fractions 17, 18, and 19 contained nearly pure *l*-β-phellandrene. Below, characteristics of fraction 18 are compared with those of β-phellandrene:

	β-phellandrene (6)	Fraction 18
n_D	1.4851^{25}	1.4860^{23}
d_4	0.8375^{25}	0.837^{22}
b. p.	70°/20 mm.	67°/17 mm.
$[\alpha]_D$	-2.86^{25}	−15.4
λ max.	232mμ	232 mμ
ε	19,300	18,200 (95% ethanol)

A 3-ml. portion of fraction 17 was used to prepare a tetrabromide by the method of Berry and MacBeth (3); 3.32 Gm. of crude tetrabromide, m. p. 104–112°, was received. After nine recrystallizations from ethyl acetate, alternated with chloroform and addition of cold methanol, 0.62 Gm. of white crystals,

m. p. 117–118°, was obtained. The tetrabromide of *l*-β-phellandrene melts at 118–119° (3).

Fractions 20 and 21 were transition fractions.

Fraction 22 was redistilled giving a heart cut (over 70% of the charge) with the following properties: b_{16}, 95–104°; d_4^{22}, 0.957; n_D^{22}, 1.5063; $[\alpha]_D^{24}$, −13.9. The properties indicate the possible presence of methyl chavicol. Since the optical rotation of methyl chavicol is 0°, it must not be pure methyl chavicol. From acid permanganate (4) oxidation of 3 Gm. of the heart cut from fraction 22 at 0°, 100 mg. of acid melting at 81–84° was obtained. It was recrystallized once from water and then melted at 84–85°. A mixed melting point determination with authentic homoanisic acid showed no depression.

To sum up, gum turpentine from *P. elliottii* var. *densa* contained: *l*- and *dl*-α-pinene, 71%; *l*-β-pinene, 3–4%; unidentified terpene, 1–2%; *l*-β-phellandrene, 19%; methyl chavicol, 3%; pot residue and loss 1.2%. It is seen from the above that chemical composition of turpentine of *P. eliottii* var. *densa* is intermediate between that of *P. caribaea* (presence of β-phellandrene) and *P. elliottii* var. *elliottii* (presence of methyl chavicol), being perhaps closer to the former.

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A Study of the Hydrolytic Action of Cascara Sagrada*

By CHARLES W. BAUER† and DOUGLAS HENRY KAY

Lipolytic and proteolytic determinations have been made on unheated 5 per cent aqueous extracts of the three lots of cascara sagrada bark. Results of experimental work indicate that cascara sagrada possesses no significant lipolytic or proteolytic-like activity. Five per cent heated and unheated extracts of the 1951 lot of cascara sagrada bark showed no significant emulsin-like activity by a modification of the N. F. IX method for the assay of bitter almond oil for benzaldehyde. The hydrolytic agent has been isolated from the 1951 lot of cascara sagrada bark as an impure, light yellow-brown residue by a modified method for the isolation of lysozyme from egg white.

IN 1888 Meier and Webber (1) stated that cascara sagrada contained a "ferment." In 1904 Jowett (2) stated that the "ferment" was emulsin. Bauer and Gosselin (3) in 1954 showed that the "ferment" has amylase-like activity, that it is not destroyed by aging for one year, and that it is not destroyed by moist heat at 100°.

A further study of this "ferment" has been made. It has been found that the "ferment" is not emulsin. It has also been found that cascara sagrada has no proteolytic or lipolytic activity but that the amylase-like activity in the bark is greatly reduced after an aging period of four years.

On the hypothesis that the hydrolytic agent in cascara sagrada might be lysozyme, since lysozyme is generally classified as an enzyme that is stable to moist heat, an attempt was made to isolate the hydrolytic agent by a modified method for the isolation of lysozyme from egg white. A brown residue was obtained by this process which had amylase-like activity 250 times greater on a weight-to-weight basis than the original bark had.

* Received April 13, 1956, from the Massachusetts College of Pharmacy, Boston.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April, 1956.
This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Douglas Henry Kay, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
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EXPERIMENTAL

The cascara sagrada used in this investigation came from the State of Washington and was from the same lot of bark used by Bauer and Gosselin in their determination of the effect of age and heat on the "ferment" present in the bark. The drug, in three lots, was collected in 1949, 1950, and 1951, respectively.[1] The effect of two years of additional storage on the activity of this hydrolytic agent was included in the investigation.

The air-dried, ground bark was placed in an Erlenmeyer flask, and distilled water was added to two-thirds of the final volume in preparing the aqueous extracts. A few drops of toluene was added as a preservative, and the drug was allowed to macerate for three days at room temperature. The aqueous extract was then filtered through a Whatman Number 1 Filter, and the final volume was obtained by adding distilled water through the filter. In those cases where heat was used, the mixture was first brought to a boil and was then allowed to simmer for five minutes before the maceration period.

Determination of Lipolytic-like Activity.—The method employed was that of Bauer and Wilson (4), in which olive oil is used as the substrate. The fatty acids, liberated as a result of lipolytic activity, are titrated with 0.1 *N* alcoholic potassium hydroxide, with thymolphthalein as the indicator. In preliminary studies it was found that the deep colors of the cascara sagrada filtrates obscured the color change of the indicator. The Beckman *p*H meter was then used, and the volume of alcoholic potassium hydroxide was noted at a *p*H of ten and of eleven in the titration.

Six milliliters of an unheated 5% aqueous extract, representing 300 mg. of the drug, was placed in both the sample and the control flasks at the point where Bauer and Wilson used pancreatin. By subtracting the number of ml. of 0.1 *N* alcoholic potassium hydroxide used in the control determination from the number of ml. required for the sample, the amount of alkali necessary to neutralize the fatty acids liberated was obtained.

The amounts of 0.1 *N* alkali used were 0.5 ml. for the 1949 lot, 0.3 ml. for the 1950 lot, and 0.2 ml. for the 1951 lot. It was concluded that the hydrolytic agent in a 5% aqueous extract of the different lots of cascara sagrada has no lipase-like activity.

Determination of Proteolytic-like Activity.—A modification of Sörensen's (5) formol titration was employed. Two and seven-tenths grams of soluble casein was placed in a mortar, and 5 ml. of cold distilled water was added to make a thin paste. One and one-half ml. of a 10% sodium hydroxide solution was added and was triturated with the paste to effect equal dispersion. The mixture was then diluted to 150 ml. with hot distilled water. Five milliliters of this mixture was removed, and was titrated to a *p*H of 8.2 with 0.1 *N* sodium hydroxide with cresol red as the indicator. The volume of the mixture was diluted to 180 ml. by the addition of sufficient 0.1 *N* sodium hydroxide (as calculated from the titration) and distilled water was added to give the final solution a *p*H reading of 8.2. This gave a 1.5% casein solution.

The digestion procedure was made as follows: Ten ml. of toluene and 180 ml. of a 1.5% casein solution were mixed in a 250-ml. Erlenmeyer flask. The mixture was then incubated in a constant temperature bath at 40°. When the temperature of the mixture reached 40°, 20 ml. of a 5% unheated aqueous extract was added and was mixed thoroughly. Immediately after being mixed 20 ml. of the solution was removed and titrated. This titration served as a control. Then at intervals of one, eighteen, and twenty-four hours additional 20-ml. portions were removed and titrated. Determinations were made on unheated aqueous extracts of the three lots of bark.

The titration procedure was done as follows: Ten drops of phenolphthalein indicator and 55 ml. of distilled water were added to the 20 ml. of digestion mixture removed. The solution was titrated with 0.1 *N* sodium hydroxide until a permanent pink tint was observed. This volume of alkali represents the acidity due to free carboxyl groups. To the solution just titrated 30 ml. of neutral formaldehyde (20%) was added, and the solution was again titrated to the same pink tint. Any acidity indicated by the second titration was due to the liberation of carboxyl groups of the amino acids liberated by proteolytic activity. The difference in the volume of alkali required for the first and the second titrations was taken as a measure of the proteolytic activity.

The amount of alkali required for the second titration was the same in all three lots for the control and for the one-, the eighteen-, and the twenty-four-hour digestion periods. It is concluded that a 5% aqueous extract of cascara sagrada does not contain an enzyme with proteolytic activity.

Determination of Emulsin-like Activity.—The procedure followed was a modification of the N. F. IX method for the assay of bitter almond oil for benzaldehyde.

According to Auld (6) the optimum conditions for the hydrolysis of amygdalin by emulsin are as follows: (*a*) There should be a ratio of five parts of amygdalin to one part of emulsin, (*b*) the reaction should be allowed to proceed for twenty-four hours at 40° in glass-stoppered containers, (*c*) the reaction mixture should have a *p*H of 7 for optimum yields.

After a series of preliminary experiments the following conditions were found to yield the best results: (*a*) toluene must be used as a preservative to prevent any increase in acidity caused by bacterial action, (*b*) the titration should proceed potentiometrically to a *p*H of 4.6 with 0.005 *N* sodium hydroxide as the titrant, (*c*) the hydroxylamine hydrochloride reagent should be ten times weaker than that specified in the N. F. IX process, since the amount of benzaldehyde that is formed is so small that the error of one drop in over-titration is too great.

Solution 1: One hundred mg. of amygdalin[2] and five drops of toluene were added to a glass-stoppered volumetric flask containing 100 ml. of an unheated 5% aqueous extract of cascara sagrada,

[1] The lots of cascara sagrada were supplied through the courtesy of Parke, Davis and Company.

[2] The amygdalin was obtained from the Delta Chemical Works, Inc., New York, N. Y.

1951 lot. The solution was shaken until the amygdalin dissolved, and it was then placed for twenty-four hours in a constant temperature bath at 40°. At the end of this time 6 ml. of the specially diluted hydroxylamine hydrochloride solution was added. The flask was returned to the bath and was shaken intermittently for fifteen minutes to allow for complete reaction. The solution was then transferred quantitatively to a 125-ml. beaker and was titrated potentiometrically with 0.005 *N* sodium hydroxide to a *p*H of 4.6.

Solution I Control: The amygdalin was omitted from this solution, which was treated exactly as was Solution I.

Solution II: This solution was treated as was Solution I, differing only in that the aqueous extract used was heated in its preparation.

Solution II Control: The amygdalin was omitted from this solution, which was treated exactly as was Solution II.

Solution III: Twenty mg. of emulsin[3] was added to 100 ml. of a 0.1% solution of amygdalin in distilled water. The odor of benzaldehyde was observed almost immediately. This solution, after the addition of the toluene, was treated exactly as was Solution I.

Solution III Control: This solution was treated exactly as was Solution III, except that the amygdalin was omitted. Results: The *p*H values of the solutions before the beginning of the titration were as follows: Solution I, 4.8; solution I control, 4.8; solution II, 4.8; solution II control, 4.8; solution III, 3.2; solution III control, 4.3.

To bring the *p*H of Solution III to 4.6 required 17.7 ml. of 0.005 *N* sodium hydroxide. Solution III control required 2.4 ml. of alkali. Therefore 15.3 ml. of 0.005 *N* alkali was required to neutralize the hydrochloric acid liberated by the action of the hydroxylamine hydrochloride on the benzaldehyde liberated from the amygdalin by emulsin. Some of this alkali also was required to neutralize the negligible amount of hydrocyanic acid liberated from the amygdalin. The 15.3 ml. of 0.005 *N* alkali was regarded as neutralizing the hydrochloric acid liberated, which is equivalent to 8.1 mg. of benzaldehyde.

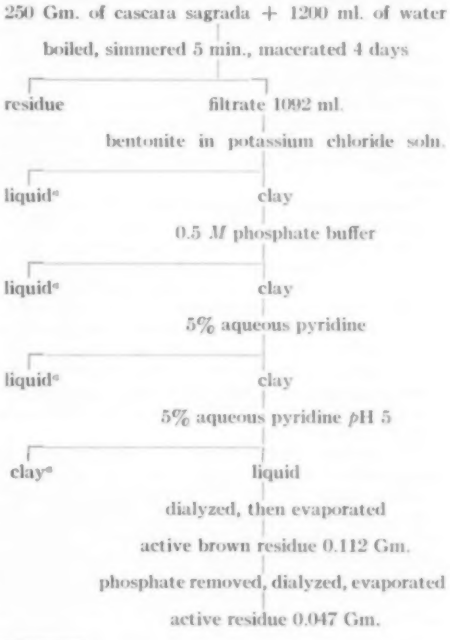
Whereas Solutions I and II attained the same *p*H (4.8) after twenty-four hours as did Controls I and II, and after comparison with the known reaction (Solution III and Control III), it was concluded that the hydrolytic agent in a 5% aqueous extract of cascara sagrada bark possesses no emulsin-like activity and that the "ferment" is not emulsin.

A Concentrated Extract of the Hydrolytic Agent.—An attempt was made to isolate the hydrolytic agent in crystalline form. The effort was unsuccessful, but a more concentrated extract was obtained. The 1951 lot was used, even though it had lost much of its amylase-like activity during the four-year storage period.

Two hundred and fifty grams of the air-dried, powdered bark was added to 1200 ml. of distilled water, which was brought to a boil, and was allowed to simmer for five minutes. The mixture was macerated for four days at room temperature; it was then filtered through folded gauze, and then through filter paper. Approximately 1090 ml. of filtrate was obtained. From this point on, a modified method of Alderton, Wald, and Fevold (7), to isolate lysozyme, a heat stable enzyme, was followed.

To a 2000-ml. flask containing the filtrate, 165 ml. of a 10% suspension of bentonite in a 1% potassium chloride solution was added. The mixture was stirred for five minutes and was then centrifuged to separate the clay.

FLOW CHART FOR THE PREPARATION OF A CONCENTRATED RESIDUE OF THE HYDROLYTIC AGENT FROM CASCARA SAGRADA BARK, 1951 LOT



[a] No activity.

The clay was first washed with 400 ml. of 0.5 *M* phosphate buffer at a *p*H of 7.5, and was then washed three times with 300-ml. portions of a 5% aqueous solution of pyridine. Elution of the active material from the clay was accomplished by washing the clay twice, each time with 400 ml. of a 5% aqueous solution of pyridine which had been previously adjusted to a *p*H of 5 by the addition of sulfuric acid.

The eluate was placed in eight cellophane bags and was dialyzed against running tap water for three and one-half days, until the odor of pyridine was absent. The dialysis was then completed against running distilled water for twenty-four hours. About 150 ml. of liquid was obtained from the dialyzing membranes.

The liquid was evaporated on a water bath almost to dryness and then was allowed to evaporate to dryness spontaneously. The dark brown

[3] The emulsin was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

residue obtained was heated to constant weight in an oven at 100°. About 112 mg. of residue was obtained. This material proved to be contaminated with calcium phosphate.

The residue was dissolved in 500 ml. of distilled water, and sufficient hydrochloric acid was added to bring the *p*H of the solution to 1.0. The solution was then dialyzed for two days against running tap water, and for one day against running distilled water. The solution from the dialyzing membranes was evaporated and was dried to constant weight as before. About 47 mg. of a shiny brown amorphous residue was obtained. This material charred at 175–180°.

This material was tested for amylase-like activity and was compared to a 5% aqueous extract of the 1951 lot. It was found that 2 mg. of this residue had the same digestion time required by 10 ml. of a 5% aqueous extract. This residue was thus about 250 times as active, on a weight-to-weight basis, as the original bark.

Effect of Storage on the Activity of the Hydrolytic Agent in the 1951 lot of Cascara Sagrada.—Bauer and Gosselin found that the lots of cascara sagrada collected in different years showed a variation in the activity of the hydrolytic agent. The most recent lot had the greatest activity and the oldest lot had the least. In their investigation they found that the concentration of the substrate and that of an aqueous extract could be adjusted so that the digestion time could be expressed in minutes.

In this investigation the 1951 lot of cascara sagrada was used; but after three additional years of aging, it was found that under identical conditions the digestion time was lengthened from seven minutes to forty-four hours.

It appears therefore that the reason the more recently harvested bark had greater hydrolytic activity than that collected at an earlier period was that the hydrolytic agent loses its activity on storage.

Comparison of Concentrated Extracts of the Hydrolytic Agent.—The concentrated extract obtained by Bauer and Gosselin was a black, shiny, glass-like substance with a melting point of about 220°. The hydrolytic activity of this residue was about 10 times greater than that of the cascara sagrada from which it was obtained.

The concentrated extract obtained by the method described in this paper chars at 175–180° and has an activity about 250 times greater than that of the bark.

It appears that the method of concentration described above affords a clue to a method that may be followed for obtaining a more nearly pure form of this hydrolytic agent.

DISCUSSION

In order to prepare the hydrolytic agent in a more nearly pure form it appears that one would obtain a much higher yield in working with freshly harvested bark; for the 1951 lot, after a period of four years' storage, was found to have lost a great amount of its amylase-like activity.

No effort was made in this investigation to relate the griping effect of cascara sagrada to the hydrolytic agent or to the age of the bark.

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Observations on the Toxicity and Antigenicity of Beef Trypsin*

By W. B. RICE and J. D. McCOLL

Guinea pigs can be sensitized to trypsin by intramuscular injections of the enzyme in oil. When challenged by an intracardiac injection or by exposure to a trypsin aerosol, these animals develop typical anaphylactic reactions which are fatal if untreated with epinephrine. No fatal anaphylactic reactions were observed following the intramuscular administration of trypsin in oil. In the rabbit, while antibody formation was evident, no fatal anaphylactic reactions were observed. The growth of rats was not significantly affected by the administration of 30 mg./Kg./day of trypsin in oil for thirteen days.

THE INTRAMUSCULAR administration of trypsin in oil has been employed clinically in the treatment of acute inflammatory states (1–3). The antiphlogistic effect of parenteral trypsin upon experimental inflammations in rats has been reported from this laboratory (4).

Achalme (5) reported that the antitryptic

* Received May 17, 1956, from the Research Laboratories, Frank W. Horner Limited, Montreal, Canada.
Read by title Canadian Physiological Society, 19th annual meeting, London, 1955.

activity of guinea pig serums was increased following pretreatment with trypsin. Broeck (6) demonstrated that pig and beef trypsins could be differentiated by the Dale anaphylactic test in the guinea pig. The present study was undertaken to investigate the antibody formation, and the response of intact rabbits and guinea pigs when challenged with trypsin by various routes.

EXPERIMENTAL

Purified crystalline beef trypsin[1] (dialyzed salt free) obtained from Princeton Laboratories, Princeton, N. J., was used. This trypsin, suspended in sesame oil (Parenzymol, Horner) was used for intramuscular injections and in saline for intravenous injections.

Beef protein was prepared by homogenizing fresh beef muscle, extracting in 0.3 *M* KCl at 5°, adjusting to *p*H 5.5, standing for two hours and then centrifuging. The supernatant was dialyzed against water for twenty-four hours and lyophilized.

Male and female guinea pigs and rabbits were injected intramuscularly with trypsin in oil (Parenzymol) for 7 days at a dose equivalent to 5 mg./Kg./day. Sixty days later these animals were challenged by intravenous or intracardiac injections of trypsin in saline (1 mg./Kg.) as well as the intramuscular injection of trypsin in oil (Parenzymol, 1 mg./Kg.). Animals similarly treated were also challenged by exposure to a trypsin aerosol (1%). A DeVilbis nebulizer with oxygen at 10 L./minute flow was employed to produce an aerosol at a rate of 0.5 ml./minute in a 10-liter chamber.

Subacute toxicity studies were made in Sprague-Dawley rats (*ca.* 200 Gm.) and in guinea pigs (*ca.* 200 Gm.).[2]

Subacute Toxicity.—The body weight gain of rats given intramuscular injections of trypsin in oil (30 mg./Kg./day) for thirteen days is shown in Table I. All groups continued to gain weight during and after the injection period. They appeared healthy and ate normally. There was no significant difference between the weights of oil-injected control and the trypsin-treated groups. Three days following the injection period the weights of all three groups were similar.

Twenty male guinea pigs (*ca.* 200 Gm.) were injected twice daily (5 mg./Kg./day) for seven days with trypsin in oil alternately into left and right thigh muscles. There was no significant difference in the weight gain of the two groups during the injection period or for a subsequent ten days. All animals remained healthy for sixty days at which time they were employed for studies on antigenicity. Histological examination of the thigh muscles showed some fibrosis in the trypsin-treated group and to a lesser extent in those which received oil.

Antigenicity.—Ten guinea pigs, repeatedly injected with trypsin for seven days with 5 mg./Kg./day, were challenged after sixty days by an intramuscular injection of trypsin in oil (1 mg./Kg.). There was evidence of an anaphylactic response in all ten animals, as manifest by a swelling and an apparent itching of the nose. There were, however, no fatal reactions following this intramuscular injection.

When these trypsin-injected guinea pigs were exposed to an aerosol of trypsin (1%) coughing attacks followed rapidly (mean time of response two minutes) and, if untreated, the animals died of acute bronchoconstriction. Increasing the concentration of the trypsin aerosol accelerated the time of onset of coughing (i. e., animals coughed at one minute with 2% trypsin).

Pretreatment of these animals with epinephrine (0.5 mg./Kg. s. c.), an antihistamine (diphenhydramine 5 mg./Kg. i. p.), or a xanthine (7-chloroethyltheophylline 70 mg./Kg. i. p.) delayed the onset of coughing after exposure to a 1% trypsin aerosol.

Intracardiac injection of trypsin (1 mg./Kg.) resulted in rapid death of these animals with terminal signs of acute bronchoconstriction. Table II summarizes these results. No fatalities were observed following the intracardiac injection of trypsin or beef protein in unsensitized animals at this dose. Similarly, no deaths resulted when trypsin was injected into those animals sensitized to beef protein and vice versa. However, in animals sensitized to beef protein or to trypsin, 100% mortality resulted following injection of the appropriate antigen. Epinephrine (1 mg./Kg. i. p.), administered ten minutes before challenging with trypsin, protected 83% of the animals tested from the fatal bronchoconstriction.

It was not possible to produce similar signs of

TABLE I.—EFFECT OF TRYPSIN IN OIL (INTRAMUSCULAR) ON THE BODY WEIGHT GAIN (GM. ± S.D.[a]) IN MALE RATS.

Group	No. of Animals	4th Day of Injections	8th Day of Injections	12th Day of Injections	16th Day, 3 Days Following Injection Period
1. Oil control	10	31.5 ± 11.8	31.0 ± 14.3	35.7 ± 16.5	46.0 ± 29.9
2. Trypsin (30 mg./Kg. i.m.)	10	26.0 ± 7.4	23.5 ± 12.0	32.2 ± 16.6	44.0 ± 17.6
"t"		1.2 N. S.	1.3 N. S.	N.S.	N. S.

[a] Standard deviation = $\sqrt{\Sigma(Xi - \bar{X})^2/N - 1}$.

[1] One mg. of this material when assayed by the hemoglobin substrate method of Anson (7) at 37°, will cause decomposition of the hemoglobin to produce TCA-soluble hydroxyaryl substances equivalent to 4.2 mg. of tyrosine. This material is approximately twice as potent as Armour crystalline trypsin.

[2] Rats were maintained on "Fox Chow Checkers" and water; guinea pigs and rabbits on "Miracle Rabbit Food," water, and fresh vegetables.

antigenicity in rabbits treated with 5 mg./Kg. trypsin/day for seven days by exposure to an aerosol of trypsin after sixty days. Trypsin-injected rabbits withstood thirty minutes exposure to 8% trypsin aerosol without any signs of discomfort, while similarly treated guinea pigs coughed within one minute in the same atmosphere.

genic reactions are not observed when crossmatched with beef protein. The lack of a fatal antigenic response following the intramuscular administration of trypsin in oil may be due to the slow release and accompanying inactivation of the enzyme from the injection site.

The rabbit will also form antibodies to trypsin as indicated by the positive precipitin response. However, the combination of the antibody-antigen in the rabbit did not produce a fatal anaphylactic response. From this it appears that the rabbit is a less susceptible species for obtaining anaphylactic symptoms. Trypsin may also act as a relatively poor antigen in the rabbit under these conditions.

TABLE II.—ANAPHYLACTIC REACTION OF SENSITIZED GUINEA PIGS TO TRYPSIN AND BEEF PROTEIN

Sensitized to	Treatment with	Challenged with		No. of Animals	Mortality, %
Trypsin	...	Trypsin	...	8	100
Trypsin	...	...	Beef protein	6	0
Trypsin	Epinephrine (1 mg./Kg.)	Trypsin	...	6	17
Beef protein	...	Trypsin	...	4	0
Beef protein	...	...	Beef protein	8	100
Control	...	Trypsin	...	6	0
Control	...	...	Beef protein	6	0

The intravenous or intracardiac injection of trypsin (1 mg./Kg.) in rabbits similarly pretreated with trypsin did not produce fatal anaphylaxis as in the guinea pig. Higher concentrations of intravenous trypsin produced death due to massive clotting in the cardiopulmonary vessels. No apparent signs of antigenicity were observed in treated rabbits when challenged by the intramuscular injection of trypsin in oil (1 mg./Kg.).

Serums for precipitin tests were obtained by cardiac puncture from both guinea pigs and rabbits. Two milliliters of the trypsin dilution was layered with 0.5 ml. of the undiluted serums. A positive reaction was obtained with concentrations of 1%, 0.5%, 0.25%, 0.125%, and 0.05% of the antigen. No essential difference, such as solubility of the precipitate in excess of antigen, was observed in these tests.

DISCUSSION

There appears to be little in the literature pertaining to the prolonged parenteral administration of trypsin in animals. Our studies indicate that a dosage of 30 mg./Kg. given repeatedly to rats for a two-week period was tolerated.

There is ample evidence that trypsin will act as an antigen in the guinea pig (5, 6). Typical antigenic responses can be shown in sensitized animals following a challenge dose of trypsin. This appears to be due to the enzyme and likely not to contamination of the trypsin with other beef protein. Anti-

The lack of reports to date of serious antigenic responses in humans following prolonged treatment with intramuscular trypsin (1) would suggest that trypsin may be a poor antigen in the human. If so, this appears to be somewhat analogous to insulin. Maloney and Coval (8) have reported that insulin was an antigen in the guinea pig but not in the rabbit. Further, they concluded from the literature that insulin appeared to be a poor antigen in the human.

Haurowitz (9) suggests that the nonantigenicity or weak antigenicity of insulin and many pituitary hormones in the human is probably due to their low molecular weight. This may also be the case with trypsin.

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The Determination of 4-Aminoquinoline Antimalarials: Revaluation of the Induced Fluorescence Method, with Specific Application to Hydroxychloroquine Analysis*

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Various technical aspects of the procedure of Brodie, *et al.*, for the determination of 4-aminoquinoline antimalarials in biological materials have been re-examined. Although it has not been possible to achieve any noteworthy increase in precision over that claimed by the original authors, the method has been materially shortened and simplified, thereby reducing the likelihood of technical errors. Specific procedures for the analysis of hydroxychloroquine are presented; the present widespread use of this compound in antimalarial therapy makes desirable the availability of an exact method for its determination in biological materials.

THE REPLACEMENT of quinacrine by chloroquine (SN-7618) as the antimalarial of choice, about ten years ago, required the development of an analytical method for compounds having the 4-aminoquinoline nucleus. Such a method was worked out by Brodie, Udenfriend, Dill and Chenkin (1), which consisted essentially of these steps: (*a*) extraction of the free base into a solvent such as ethylene dichloride or petroleum ether, (*b*) removal of metabolic products of the drug, usually by washing the solvent with dilute alkali, (*c*) transfer of the base from the solvent into dilute acid, (*d*) buffering at *p*H 9.5, and (*e*) development of fluorescence by U. V. irradiation. The kinetics and nature of the changes which occur during U. V. irradiation have been studied by Price, Jackson, and Pohland (2), who suggested that there is a transformation of the 4-aminoquinolines into compounds of the anthranoylacetaldehyde type. Fuhrmann (3) has published a turbidimetric method for the determination of chloroquine[1] (CQ) in urine, based on its precipitation by potassium mercuric iodide. We have found that a concentration of at least 6 mg. per liter of CQ is required to give a detectable turbidity, while hydroxychloroquine[2] (HCQ) gives no turbidity at concentrations less than 40 mg. per liter, and the metabolite of CQ, SN-13,616, (4, 5) requires a concentration of at least 50 mg. per liter. This method, therefore, can have at best a very limited application to the problem of analysis for the 4-aminoquinolines; namely, for the detection of high concentrations of CQ in urine (6).

EXPERIMENTAL

According to the Brodie group, the recovery of CQ from serum or plasma by their method, in the range of 0.2 to 0.6 μg. in the sample taken, is of the order of 98 ± 5.5%. In our experience, 92 ± 8% would be a better estimate; however, the latter figures are based on comparisons against standards carried through the irradiation step only. If the same data were evaluated against standards carried through both extraction and irradiation procedures, the recovery would be of the order of 98 ± 9%. In other words, our findings differ from those of the Brodie group only in the magnitude of the standard deviation. Many hundreds of determinations of CQ have been carried out in this laboratory by the Brodie method, and it has generally proved to be satisfactory. Occasionally, however, extremely erratic recoveries, averaging 70 ± 20% (measured against standards carried through the irradiation step only), have been obtained. It has not been possible to fix the responsibility for the poor result on any item involving technique or materials and it has, therefore, been very disturbing. The most probable explanation of this phenomenon seems to be the marked tendency of the free CQ base to be adsorbed on glass surfaces from petroleum ether solutions. The Brodie group recognized this problem and tried to overcome it by adding a few drops of ethanol, in a very exactly prescribed way.[3] However, there must always be some doubt in the mind of the analyst as to the efficacy of this operation, and the precision of the whole method can be no greater than that of any one step. In view of the unexplained aberrations which have characterized our experience with the procedure, and the fact that the metabolism of the 4-aminoquinolines had not been worked out in detail

* Received April 16, 1956, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

[1] Available from Winthrop Laboratories under the tradename of Aralen.

[2] Available from Winthrop Laboratories under the tradename of Plaquenil.

[3] Alving and co-workers (7) added the alcohol in definitely measured volumes, directly to the petroleum ether, at two specific points in the procedure.

TABLE I.—CALCULATED AMOUNTS OF ANTIMALARIALS AND OF THEIR METABOLIC PRODUCTS REMAINING IN ETHYLENE DICHLORIDE AFTER ONE EXTRACTION WITH AN EQUAL VOLUME OF AN AQUEOUS BUFFER SOLUTION OF THE INDICATED *p*H

Chloroquine (*p*H 7.85)			Hydroxychloroquine (*p*H 8.25)		
Unchanged Drug Present, mg.	Metabolite Present, mg.	Total in Solvent Phase, mg.	Unchanged Drug Present, mg.	Metabolite Present, mg.	Total in Solvent Phase, mg.
100	0	92.0	100	0	94.0
90	10	85.7	90	10	87.5
80	20	79.4	80	20	81.0
70	30	73.1	70	30	74.5
60	40	65.9	60	40	67.0
50	50	60.5	50	50	61.4

when the Brodie procedure was developed, it has seemed advisable to re-examine the entire problem in a critical way.

For most purposes, ethylene dichloride is a more suitable solvent for the analysis of the 4-aminoquinolines than is petroleum ether. It is much less likely to present difficulties with adsorption of the bases on glassware, it constitutes no fire hazard, and, since it forms the lower layer, repeated aspirations of the aqueous phase are more readily accomplished. Ethylene dichloride has the disadvantage that it is obviously less selective in its solvent action than petroleum ether. The Brodie group did suggest it for the determination of SN-7135 and, in fact, some such solvent must be used for the analysis of HCQ, the free base of which does not pass quantitatively from water into petroleum ether at any *p*H level. The availability of authentic samples of SN-13,616 and of the principal metabolite of HCQ (7-chloro-4-[4-(2-hydroxyethyl)-1-methylbutylamino]quinoline) has made it possible to study the distribution of the two antimalarials and of their metabolic products between ethylene dichloride and aqueous buffer solutions (5). At *p*H 7.85 the approximate distribution constant of CQ is 0.09, while that of SN-13,616 is 2.5. Since HCQ is more hydrophilic than CQ, a higher *p*H is required to force it into the organic phase: at *p*H 8.25 its distribution constant is about 0.06, while that of its metabolite is again 2.5. These marked differences in distribution make it possible to achieve a satisfactory separation of the unchanged drugs from their metabolic products in one washing operation (Table I), as compared to the two washing operations used in the Brodie procedure. Furthermore, the Brodie method is not directly applicable to the analysis of HCQ, since the washing of an ethylene dichloride solution of this drug and its metabolite, with 0.1 *N* alkali, removes neither compound. To remove most of the metabolite requires washing with an aqueous buffer solution having a *p*H low enough to remove a small amount (i. e., at least 5%) of the unchanged drug. The figures given in Table I demonstrate that, within the expected range of composition of most biological materials (0 to 40 mol. % of metabolite), one extraction of the ethylene dichloride with one volume of the appropriate buffer solution will lead to results for CQ and HCQ which are well within the usual requirements of accuracy for such materials (±10%). The small amount of metabolite which remains in the solvent phase compensates almost exactly for the unchanged drug which is removed by the buffer solution. Two complete washing operations, therefore, are unnecessary.

In the course of this work all phases of the analytical procedure have been examined as potential sources of error. If adsorption on the glassware can be eliminated as a hazard, the precision of the irradiation process comes most into question. To examine this, four sets of CQ standards were prepared covering the range 0.1 to 0.4 μg., each concentration being made up in quadruplicate. The samples were buffered at *p*H 9.5 in the presence of cysteine, as in the Brodie method. They were read in the fluorometer (Coleman, model 12C) before irradiation, then read again after 60, 90, and 120, minutes of irradiation. The mean induced fluorescence (against quinine sulfate, 0.016 mg. per liter, set at 90 scale divisions) was calculated for the entire series, in terms of scale divisions per μg., and the S. D. of the 16 individual samples from this mean was determined, with these results: at sixty minutes, 166 ± 10; at ninety minutes, 188 ± 9; and at one hundred and twenty minutes, 220 ± 10. These standard deviations are respectively, 6, 4.8, and 4.5%. Since the purpose of adding the cysteine is said to be to remove oxygen from the solutions (1), it seems that there should be some advantage in keeping the cuvettes stoppered from the time the cysteine-buffer reagent is added, to prevent further access of air.[4] This was done, in a similar series of 16 standards, with these results, as net scale divisions per μg.: sixty minutes, 162 ± 7; ninety minutes, 191 ± 7; one hundred and twenty minutes, 202 ± 6. These standard deviations are, respectively, 4.3, 3.7, and 3%. Stoppering the cuvettes, therefore, results in an increase of precision of about one-third. It would seem that this precaution is worth while, and that under these conditions two hours of irradiation will give the most consistent results. The optimal time, of course, would depend on the age and output of the U.V. lamp (in this work a GE S-4 lamp was used). The importance of minimizing the error at the irradiation step is evident from the fact that the sample at this point usually represents only about half of the starting material. It is our practice, as indicated above, routinely to read the samples in the fluorometer before irradiation. This represents very little extra work, and it serves to demonstrate that no fluorescent material has been introduced acci-

[4] Price, Jackson, and Pohland (2) have suggested that the purpose of the cysteine is to provide internal screening against lower wavelength radiation, thereby retarding the radiation-induced degradation of the fluorophore.

dentally. The results are calculated from the amount of fluorescence induced in standards and unknowns (see formulas below).

A significant simplification of the procedure of Brodie, *et al.*, is possible at step (*d*); namely, the point at which the final addition of reagents is made to the 5 cc. of 0.1 *N* hydrochloric acid solution. The original paper calls for the successive addition of three reagents: 1 cc. of 0.5 *N* alkali, 1.5 cc. of a buffer solution of *p*H 9.5, and 0.5 cc. of a neutral cysteine solution. Obviously, these three reagents could be combined into one.[5] It could be prepared according to either of the following formulas:

Formula A: cysteine hydrochloride, 835 mg.; borate buffer solution (Brodie formula), 50 cc.; sodium hydroxide, 2.2 *N*, 10 cc.; distilled water, 40 cc.

Formula B: sodium sulfite anhydrous, 10% solution, 10 cc.; borate buffer solution, 50 cc.; sodium hydroxide, 2.2 *N*, 7.5 cc.; distilled water, 32.5 cc.

Formula A is prepared in the required amount just before it is to be used, but Formula B should be stable for longer periods of time. In our experience Formula B actually gives better results: when it is used, the optimal time for irradiation is ninety minutes, and for standards run in quadruplicate (range 0.1 to 0.4 μg.) the S. D. from the mean within a group of 4 is less than ±1 scale division of induced fluorescence. Either reagent is added in the proportion of 3 cc. for 5 cc. of 0.1 *N* hydrochloric acid; the resultant *p*H is 9.5, and an adequate amount of the stabilizer is provided. To use one of these combination reagents simplifies the procedure materially, and makes it virtually impossible for the analyst to add the wrong amount of any reagent, or accidentally to omit a reagent entirely. If any error is made, all samples and standards will usually be affected in the same way (barring the possibility of adding the wrong amount of the buffer-stabilizer reagent, and this error would be visually apparent).

Proposed Analytical Procedure.—To the plasma sample (2 to 10 cc., depending on the anticipated antimalarial level) in a 50-cc. glass-stoppered graduated cylinder add an equal volume of 0.1 *N* sodium hydroxide in 20% sodium chloride, 30 cc. of ethylene dichloride (purified according to Brodie, *et al.*), and shake for thirty minutes. Draw off the supernatant as completely as possible, making sure to remove all traces of emulsified protein, add about 10 cc. of water, shake a few times by hand, and aspirate off the water. Add an amount of buffer solution[6] equal to that of the remaining solvent phase, and shake mechanically for five minutes. Aspirate off the aqueous phase, and transfer exactly 20 cc. of the solvent into a clean[7] 25-cc. glass-stoppered graduated cylinder containing 6.5 cc. of 0.1 *N* hydrochloric acid. Shake for five minutes, then transfer 5 cc. of the acid phase to a fluorometer cuvette. Add 3 cc. of the cysteine-buffer or sulfite-buffer reagent described above, and stopper. Mix, and read the fluorescence against an appropriate quinine setting (filter system B_1-S, PC-1). Half an hour after adding the buffer solution put the samples in the irradiator, and irradiate for ninety minutes.[8] Read the fluorescence at the same quinine setting. Standards containing 0–0.8 μg. (as base) are irradiated at the same time as the unknowns, along with a reagent blank which has been carried through the entire extraction and irradiation procedures.

Calculation.—Subtract from the standard readings any change in fluorescence of the standard blank, and subtract from the unknowns any change in fluorescence of the reagent blank. Calculate the mean induced fluorescence per μg. of antimalarial, then: (Induced fluorescence of unknown) × 1.95/-(Mean induced fluorescence per microgram of a standard) × (volume of sample) = μg. base/cc. plasma.

Recoveries of Chloroquine and Hydroxychloroquine from Water and Blood Serum.—Quantities of 0.1 to 1 μg. of CQ added to water, in duplicate (total of 12 samples), were recovered to the extent of 91.6 ± 6%. The accuracy at 0.1 μg. was as satisfactory as at 1 μg. Since about 8% of the CQ is removed by the buffer solution, an overall recovery greater than 92% is not to be expected. Similarly, for HCQ, the recovery for amounts ranging from 0.2 to 0.8 μg. (each in triplicate, a total of 12 samples) was 99.5 ± 9%. This was about 5% more than the expected recovery, and was occasioned mostly by high percentage recoveries at the 0.2 μg. level. Two separate recovery experiments were carried out on CQ added to 4 cc. of blood serum. In one, adding amounts of 0.1 to 1 μg. (12 samples), the recovery was 94.4 ± 9.8%; in another experiment which was similar except that the range covered was 0.06 to 0.6 μg., the recovery was 94.5 ± 9.2%. The mean recovery in each case was in the expected range, but it seemed to be impossible by any improvement in the technique o obtain an S. D. below 9%. For HCQ added to serum at levels ranging from 0.1 to 1 μg. (a total of 12 samples), the recovery was 91 ± 9.5%. The omission of one sample at the 0.1-μg. level would have given a mean recovery of 92.5 ± 6%. Considering the known distribution of HCQ between solvent and buffer solution, the expected recovery is 94.5%.

Demonstration that the Proposed Method Distinguishes Between Antimalarials and Their Metabolic Products.—When this phase of the work was reached, authentic samples of the metabolites of CQ and HCQ were no longer available; therefore it was necessary to obtain solutions containing them by passing the parent drugs through animals. The composition of the urine samples in terms of unchanged drug and metabolic product was established by the following technique, which is in essence an abbreviated countercurrent distribution method (8):

An amount of urine containing about 100 μg. of total fluorophore was made alkaline by adding an

[5] Alving and co-workers (7) accomplished somewhat the same purpose by combining the buffer and alkali reagents into one, but they added the cysteine solution separately.

[6] Composition: Dipotassium phosphate and boric acid, each 0.1 *M*, and sodium chloride, 1.7 *M*. Adjust electrometrically to *p*H 7.85 or 8.25, depending on whether the analysis is for CQ or HCQ.

[7] It is vitally important that these cylinders should be scrupulously clean. We have found that routine laboratory cleansing procedures are not adequate, and that traces of detergent remaining in the cylinders can lead to very erroneous (usually low) results. Washing them once with distilled water, followed by methanol, removes the contaminants.

[8] If the irradiator is started about fifteen minutes before the samples are introduced, the time of irradiation can be shortened. Under these conditions ninety minutes would be optimal with buffer formula B (sulfite) and one hundred five minutes with formula A (cysteine).

equal volume of 0.1 *N* sodium hydroxide in 20% sodium chloride and extracted with 25 cc. of ethylene dichloride (purified according to Brodie, *et al.*). The aqueous phase was aspirated off, and the solvent washed once with 10 cc. of water. An aliquot of 1 cc. of solvent was transferred to a 25-cc. glass-stoppered graduated cylinder containing 15 cc. of 0.1 *N* hydrochloric acid, and this was set aside for subsequent analysis. The solvent was then extracted with an equal volume of the appropriate buffer solution (*p*H 7.85 for CQ: *p*H 8.25 for HCQ), and another aliquot of 1 cc. of solvent was removed for analysis. After each step the buffer solution was aspirated off and replaced by a volume of fresh buffer solution equal to that of the solvent then remaining. This was repeated until at least 6 extractions had been made. A parallel experiment on about 100 μg. of the pure drug in 25 cc. of the solvent gave the rate at which it was being removed from the solvent by the buffer solution. Five cubic centimeter-aliquots of the acid phases from each step were analyzed in the usual way, and the results were calculated in terms of the original volume of solvent. The results of such an experiment are given in Table II. The urine was obtained from a subject who took one CQ tablet (150 mg. base), and collected urine for twelve hours thereafter. The CQ content of the urine may be calculated mathematically as shown in the table, or by plotting the results on semilog paper and extrapolating back to zero washes (Fig. 1). From a complete 25-plate countercurrent distribution (9) of the same urine sample the proportion of CQ (peak concentration in tube 1) to metabolite (peak concentration in tube 18) was estimated as 82:18. Attention is called to the fact that the amount of fluorophore remaining in the urine extract after one washing with the buffer solution (Table I) is well within the mean ±S. E. of its calculated CQ content. This offers the possibility of determining both parent drug and metabolite, in the same extract, by analysis of two aliquots of the ethylene dichloride. One aliquot is washed with the buffer solution of *p*H 7.85, and the other with the buffer solution of *p*H 9.5; the latter will remove neither parent drug nor metabolite.

TABLE II.—SIMPLIFIED COUNTERCURRENT DISTRIBUTION ANALYSIS OF CHLOROQUINE AND OF URINE FROM A SUBJECT WHO INGESTED CHLOROQUINE

No. of washes at *p*H 7.85	Chloroquine			Chloroquine Urine (2 cc.)		
	Micrograms Found	Remaining in Solvent, %	Removed per Wash, %[a]	Micrograms Found[b]	Remaining in Solvent, %	Chloroquine, Calculated, %[c]
0	102.5	100.0	...	111[e]	100.0	...
1	96.8	93.6	6.4	92.5	83.4	...
2	89.5	87.3	6.5	84.0	75.8	89.5
3	75.8	74.1	9.5	71.0	64.0	82.2
4	...[d]	...	...	64.5	58.2	81.2
5	66.0	64.4	8.4	60.5	54.4	82.5
6	55.6	55.2	9.4	58.3	52.5	85.4
		Mean ±S. E. = 8.0 ± 2.1			Mean ±S. E. = 84.4 ± 1.5	

[a] Method of calculation: $\left(1 - \sqrt[n]{A/100}\right) \times 100 = B$, where n = number of washes; A = % remaining after n washes; and B = % removed per wash.
[b] Total fluorophore, calculated as CQ.
[c] Method of calculation: $A/(100 - B/100)^n$ = % present at zero washes (i. e., the % CQ).
[d] Sample lost.
[e] Found by the original Brodie procedure (1), 97 μg.

Some typical biological materials were now analyzed, with the results given in Table III. The extracts of rat liver were prepared as follows: the animal was killed two hours after the last of three oral doses of the drugs, which totaled 10 mg. per Kg. of the base (CQ or HCQ) in twenty-four hours. The entire liver was homogenized in 100 cc. of 0.1 *N* hydrochloric acid, to which was added 15 Gm. of sodium chloride, 2 cc. of 10 *N* sodium hydroxide, and 250 cc. of ethylene dichloride. This mixture was shaken mechanically for thirty minutes, after which

TABLE III.—ANALYSIS OF SOME TYPICAL BIOLOGICAL MATERIALS FOR CHLOROQUINE AND HYDROXYCHLOROQUINE BY THE SIMPLIFIED COUNTERCURRENT DISTRIBUTION METHOD

Species	Material Analyzed	Drug Administered	Total Fluorophore Present[a]	Amount Present After One Buffer Wash[b]	Unchanged Drug Present[c]
Dog	Urine	HCQ	89.5	68.2	67.5 ± 0.97
Man	"	"	6.3	5.7	5.5 ± 0.13
"	"	"	7.4	6.5	6.8 ± 0.18
"	"	CQ	7.7	5.8	6.2 ± 0.25[e]
Rat	Liver[d]	HCQ	38.1	24.4	24.0 ± 0.57
"	"	CQ	31.5	25.4	26.0 ± 0.72

[a] As micrograms per cc. of urine or Gm. of fresh liver.
[b] Calculated back to the original volume or weight, in same terms as (*a*).
[c] By mathematical calculation from the results of a series of washes, as in Table II. Values are as micrograms per cc. or Gm., ±S. E.
[d] The ethylene dichloride extract was prepared as described in the text.
[e] Found by the original Brodie procedure (1), 7.2 micrograms per cc.

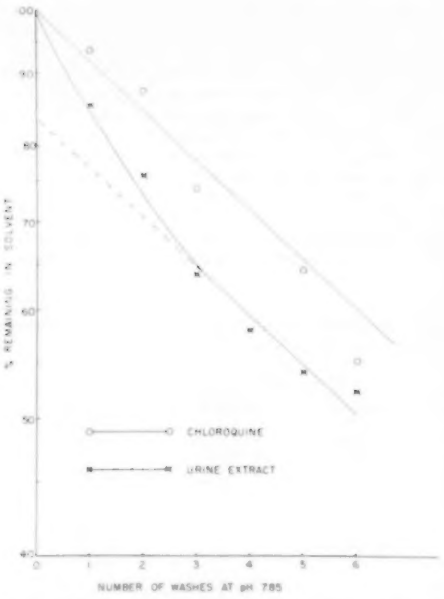

Fig. 1.—Results of analysis of a urine sample by repeated washings of its ethylene dichloride extract with an aqueous buffer solution. Extrapolation of the curve to zero washes gives the amount of unchanged drug in the sample.

it was centrifuged and the solvent phase was drawn off. This was washed once with an equal volume of aqueous buffer of *p*H 9.5, to remove greenish fluorescent materials (5), then an aliquot of the solvent was removed for analysis. The solvent was subsequently washed 8 times with equal volumes of an aqueous buffer solution of the appropriate *p*H (7.85 or 8.25) and aliquots of solvent were removed for analysis after each wash. The original content of the solvent in terms of unchanged drug was then calculated, as in Table II.

These results demonstrate again the principle that one extraction of the ethylene dichloride phase with an equal volume of the appropriate buffer solution leaves in the former an amount of fluorophore which approximates very closely the actual quantity of unchanged drug present, as may be shown by further extractions of the solvent with the same buffer. The data in Table III also give some information about the rate of metabolism of the two antimalarials in man and animals. Thus, in man (see also Table II) urine collected for the first twelve hours after the ingestion of CQ contains about 20% of metabolite and 80% of unchanged drug. In contrast, two other subjects who took the equivalent dose of HCQ showed, for the same period after medication, only an 11% conversion to the dealkylated derivative. In the dog, urine collected for twenty-four hours after the ingestion of HCQ contained 24% metabolite, 76% unchanged drug. Based on the analysis of liver extracts, it would appear that the rat metabolizes HCQ more rapidly than it does CQ.

Finally, analyses were made of dog plasma samples collected before, and ninety minutes after, the intramuscular injection of HCQ (12 mg. per Kg.) as the diphosphate. This drug was chosen because the dog can tolerate a much larger dose of HCQ than it can of CQ, and very high blood levels can, therefore, be obtained (10). Various amounts of HCQ were added to the plasma samples obtained from the dogs prior to medication and, with one possible exception, these additions were satisfactorily recovered. The mixture of HCQ and metabolite obtained from the dog urine (Table IV) was also added to some of the samples, and satisfactory recoveries in terms of HCQ were obtained, demonstrating that the added metabolite was removed by the buffer extraction. Similar additions of HCQ and of metabolite were made to the plasma samples obtained ninety minutes after medication, and all were satisfactorily re-

TABLE IV.—RECOVERY OF HYDROXYCHLOROQUINE FROM 2 CC. OF DOG SERUM WHEN ADDED AS HCQ OR AS A MIXTURE OF HCQ AND ITS METABOLITE

Sample[a]	Additions, μg. HCQ	Additions, μg. Metabolite	Found, as HCQ, μg.	Recovery of Added HCQ, %
1	1	0	0.918	91.8[d]
1	2	0	1.81	90.5[d]
1	1.93[b]	0.62[b]	1.79	92.8
2	1	0	0.90	90.0[d]
2	2	0	1.75	87.5[d]
2	0.965[c]	0.31[c]	0.95	98.4
3	0	0	0.89, 1.03	...
3A	1	0	2.02, 2.02	106, 106
3B	0.965[c]	0.31[c]	1.94, 2.00	101, 101
3C	1.93[b]	0.62[b]	2.84, 2.96	98, 102
4	0	0	0.73, 0.86	...
4A	1	0	1.73, 1.81	97, 101
4B	0.965[c]	0.31[c]	1.68, 1.75	96, 99
4C	1.93[b]	0.62[b]	2.62, 2.71	96, 99

[a] Samples 1 and 2 were taken from two dogs before any medication was given. Sample 3 was taken from dog 1 and sample 4 from dog 2, ninety minutes after the intramuscular injection of 20 mg. per Kg. of HCQ diphosphate. Samples 3A–3C represent various additions to sample 3, and 4A–4C, similar additions to sample 4. The last 8 listed samples were all analyzed in duplicate, and the recoveries for 3A–3C and 4A–4C were calculated on the assumption that the mean values obtained for samples 3 and 4 are correct.

[b] The addition was 2 cc. of a 1:70 dilution of the dog urine described in Table II.

[c] The addition was 1 cc. of the same 1:70 dilution.

[d] Since about 5% of HCQ is removed by the one washing of the solvent by the buffer solution, a recovery of over 95% is not to be expected.

covered. However, the quantities of plasma, and the concentrations of drug therein, were not sufficient to permit their direct analysis in terms of unchanged drug and metabolite, as in Table II. The results of the addition experiments demonstrate that the basal values (450 to 500 μg. per liter) must be essentially correct.

CONCLUSIONS

The induced fluorescence method of Brodie, *et al.*, for the analysis of 4-aminoquinoline antimalarials has been re-examined in detail, with special reference to the problem of determining hydroxychloroquine (HCQ) in biological materials. Although it has not proved possible to increase materially the precision claimed for the original procedure, the following significant technical simplifications have been demonstrated as feasible and have been incorporated into a proposed modified procedure:

(*a*) No special precautions against adsorption of the free base on the glassware are necessary.

(*b*) The extraction and washing operations may be carried out in the same container.

(*c*) Only one complete washing operation is necessary; the amount of fluorophore then remaining in the solvent very closely approximates the original content of the extract in terms of unchanged drug. Analysis of separate solvent aliquots, one extracted with the appropriate buffer solution and the other not so treated, permits an estimation of its content of both unchanged drug and metabolite.

(*d*) By suggested modifications of the conditions of irradiation, the magnitude of the error at this point has been reduced to a minimum.

(*e*) The cysteine-buffer solutions may be added as a single reagent, instead of as three separate solutions.

(*f*) It is not necessary to run standards through the entire extraction procedure, except as an occasional check on the accuracy of the method.

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Modification of Morphine, *l*-Isomethadone, *dl*-Alpha Acetylmethadol, and *n*-Allylnormorphine Constipation by Hydergine*,[1]

By W. C. VAN ARSDEL III and N. A. DAVID

Small nonadrenolytic doses of Hydergine® (0.06 mg./Kg.) given before morphine in the rat and rabbit further increased constipation but did not significantly alter the intense constipation caused by *l*-isomethadone, *n*-allylnormorphine, and *dl*-alpha acetylmethadol. Hydergine, alone, depressed intestinal motility in both the rat and rabbit. In some rabbits it elicited a prompt defecatory response to give an increased scybala count for the first hour period of observation. By making periodic scybala counts in comparison with Eddy's procedure of measuring the percentage of rabbits failing to defecate after injection, the overall effect of small doses of Hydergine was to slightly increase analgetic constipation. These effects of nonadrenolytic doses of Hydergine (0.06 mg./Kg.) are probably due to central stimulation.

HYDERGINE®, now available clinically as a vasodilator and antihypertensive agent, is a mixture of dihydrogenated ergot compounds. Pharmacologically it is a potent adrenolytic, acting through both a central and peripheral mechanism. We have found that small doses of Hydergine® (0.06 mg./Kg.), not believed to be adrenolytic, modify some of the autonomic responses elicited by analgetic drugs. The dose of 0.06 mg./Kg. of Hydergine® blocks mor-

* Received October 27, 1955, from the Department of Pharmacology, University of Oregon Medical School, Portland.
[1] Hydergine is the Sandoz trade name for an equimixture of dihydroergocornine, dihydroergocryptine, and dihydroergocristine.
The authors wish to thank Mr. Harry Althouse, Mgr., West Coast Office, Sandoz Pharmaceuticals, Inc., San Francisco, Calif., for support in this study.

phine hyperglycemia in the rabbit (1), potentiates morphine analgesia in rats (2), and when morphine is injected daily with Hydergine® in rabbits and the hyperglycemic responses are followed the development of morphine tolerance is delayed (3).

Constipation caused by morphine is associated with changes in autonomic nervous system activity. Vaughan Williams and Streeten (4) claim that morphine excites increased neurotropic activity of the intestines through stimulating both the sympathetic and parasympathetic systems and that this is a major factor in causing constipation. Since small doses of Hydergine® alter other autonomic responses induced by morphine we were interested in finding out if they could modify morphine constipation. We hoped, also, to throw some light on the mechanism of action concerned here.

The present paper offers further data (5) on these problems and deals with a comparison of methods used to study the effects of drugs on intestinal activity in small animals.

EXPERIMENTAL

We used a modification (6) of Macht's method to measure the distance traversed by an intubated charcoal meal in the rat's intestine within a certain period of time following drug injection. Adult Sprague-Dawley rats, fasted overnight, were injected subcutaneously with saline or the drug. One-half hour later the animals were intubated with 1 ml. of a carbon-black tragacanth mixture (5% of each) and an hour later the rat was killed by a blow on the head. The intestines were immediately excised from the pylorus to the anus and carefully laid out on a paper towel for measurement. The distance from pylorus to anus, as well as the distance traversed by the charcoal meal (expressed as the percentage of the total length of the intestines) was measured. When Hydergine® was used it was injected one-half hour before the analgetic drug.

The other method employed was a modification (7) of Eddy's procedure for studying intestinal activity in rabbits by doing periodic scybala (fecal) counts. Groups of about 16 adult albino rabbits were acclimated in individual screen-bottom cages, fed a "dry" diet of Purina rabbit chow and allowed vegetable greens once a week. Previous experience had shown that night-time "counts" were more constant and reliable than daytime ones since only formed scybala were passed during the night. Therefore, observations were made from 5:00 p. m. to midnight, or later. Control studies using saline were made two or three days each time before studying the effects of drugs. Morphine or the synthetic analgetics were usually given at 5:00 p.m. with either Hydergine® or saline administered twenty minutes previously. Scybala were then collected and counted at one, three, five, seven, and occasionally, fifteen hours thereafter. The average number of scybala passed per hour was determined by dividing the number of hours in the collection period into the average of the total number of scybala. A week's rest period was allowed between drug trials.

The analgetic drugs used were morphine sulfate and the hydrochlorides of *l*-isomethadone, *dl*-alpha acetylmethadol, and *n*-allylnormorphine.[2] Although different dosages of Hydergine® were used with the analgetics we were mainly interested in the effects produced by 0.06 mg./Kg. of Hydergine®.

Table I.—Effect of Hydergine® when Given Before or After Saline on Average Distance Traversed by Carbon Meal in Rat's Intestine

Drugs and Procedure	mg./Kg.	No. Rats Used	Aver. % Distance Traversed by Meal, %	S. E.	No. of Pyloro-spasms
Saline (control) 1/2 hr. before charcoal meal	(2 ml.)	233	64	0.7	0
Hydergine, 1/2 hr. before charcoal meal	0.03	10	64	2.9	0
	0.06	10	50.4	3	0
	0.12	10	55.6	2.1	0
	0.24	5	43.3	4.5	1
Hydergine 1 hr. and saline 1/2 hr. before meal	0.06	8	58.2	3.5	0
	0.24	17	53.4	2.4	0
	0.48	17	52.8	3	0
	0.96	10	56.1	2.1	0
Saline 1 hr. and Hydergine 1/2 hr. before meal	0.06	7	51.4	3.4	0

RESULTS

By averaging the data obtained from many groups of from 5 to 10 rats the average per cent distance traversed by the charcoal meal was found to be 64% for 233 saline-injected animals. Hydergine® had little effect on intestinal activity and when given one hour instead of the usual one-half hour

[2] The synthetic analgetics *l*-isomethadone and *dl*-alpha acetylmethadol and the morphine antagonist *n*-allylnormorphine (Nalline®) were kindly supplied through the courtesy of Merck and Company, Rahway, N. J.

before intubation even less effect was noted as shown in Table I.

Table II shows the effects of morphine or *l*-isomethadone on the rat's intestinal activity when given alone or after various dosages of Hydergine®. The average per cent distance traversed by the meal with 2 mg./Kg. of *l*-isomethadone was 42.7% and with the same dose of morphine it was 30.1%. Prior injection of either 0.06 or 0.96 mg./Kg. of Hydergine® further decreased motility. With the 2 and 4 mg./Kg. doses of *l*-isomethadone a very slight increase in effect was noted when preceded by 0.06 mg./Kg. of Hydergine®. Larger doses of Hydergine® slightly lessened the marked constipative effect of *l*-isomethadone, possibly through an adrenolytic action.

We have noted that following administration of analgetic drugs in the rat the carbon meal occasionally fails to pass the pylorus. With higher doses the incidence of this effect increases (Table II). Since this phenomenon was never observed in control rats and occurred in only one rat given Hydergine® it could hardly be due to trauma on intubation. Presumably it was caused by the analgetic acting directly or through autonomic influences to clamp off the pylorus. For example, when 43 rats were given 2 mg./Kg. of morphine complete retention of the meal was noted in 11 (25.6%). But no "pylorospasms" were noted when 0.06 or 0.96 mg./Kg. of Hydergine® preceded this dose of morphine. With both doses of *l*-isomethadone the incidence of expected pylorospasms was increased by prior administration of 0.06 mg./Kg. of Hydergine® and decreased by higher doses of the latter drug.

Control scybala counts following saline injection to rabbits were usually made two or three days before each drug experiment. It was impossible to obtain average scybala counts for the several collection periods since the counts varied depending upon the time of year they were done. During the late spring and summer when the advancing daylight hours changed the feeding and activity habits of the rabbits, control values also changed. We have, therefore, used data obtained for the average control scybala count during the winter in most of these studies as had been done in previous work (7).

Single doses of 0.03 mg./Kg. of Hydergine® decreased the scybala count during the second (3-hour) collection period while larger doses of 0.06 and 0.12 mg./Kg. decreased the counts beyond this period (Fig. 1). The reason for the increased average count noted at the end of the first hour collection period with 0.12 mg./Kg. of Hydergine® was because an immediate defecatory response resulted following injection.

When 0.06 mg./Kg. of Hydergine® is given before 1 mg./Kg. of morphine further reduction in the scybala count occurs (Fig. 2). This reduction was about equal to that noted for 2 mg./Kg. of morphine alone. With 0.06 mg./Kg. of Hydergine® plus the 2 mg./Kg. dose of morphine a prompt defecatory response was observed during the first hour with the other changes differing but little from those caused by 2 mg./Kg. of morphine alone. Prior injection of 0.06 mg./Kg. of Hydergine® to 1 mg./Kg. of *l*-isomethadone appeared to double the constipative effects of the latter drug (Fig. 3). Similarly, when Hydergine® was given before 0.5 mg./Kg. of *dl*-alpha acetylmethadol and 2 mg./Kg. of *n*-allylnormorphine the effects approximated those with 1 mg./Kg. of *dl*-alpha acetylmethadol and 4 mg./Kg. of *n*-allylnormorphine given alone (Fig. 4).

If, instead of making periodic scybala counts, one employs Eddy's (9) original method of measuring the *percentage of rabbits failing to defecate* during the collection periods somewhat different information is provided. This is especially noticeable when data for the first hour observation period for both methods are compared. For example, Table III shows that 0.12 mg./Kg. of Hydergine® increased the percentage of nondefecating rabbits from the saline control of 21% to 31% for the first hour period suggesting a mild constipative effect. But when scybala counts were made (Fig. 1) the counts were greater for this period. This difference can be ex-

TABLE II.—EFFECTS OF HYDERGINE, MORPHINE AND *l*-ISOMETHADONE ON AVERAGE DISTANCE TRAVERSED BY CARBON MEAL IN RAT'S INTESTINE

Hydergine Dose, mg./Kg.	Morphine or *l*-Isomethadone Dose, mg./Kg.		Total No. Rats Used	Average Distance Traversed By Meal, % — Distance, av. %	No. Rats	S. E.	Pylorospasms — No. Rats	Total, %
..	(Saline)	2 ml.	233	64	233	0.7	0	0
..	Morphine	2	43	30.1	32	2.6	11	25.6
0.06	"	2	10	20.5	10	2.4	0	0
0.96	"	2	10	29.4	10	3.7	0	0
..	"	4	14	23.6	13	2.6	1	7.1
0.96	"	4	10	20.1	10	2.7	0	0
..	*l*-Isomethadone	1	37	51.1	36	2.2	1	2.7
0.06	"	1	8	47.7	8	5.3	0	0
..	"	2	42	42.7	40	2.7	2	4.7
0.06	"	2	10	38.3	6	8.8	4	40
0.12	"	2	10	40.8	9	4.4	1	10
0.24	"	2	10	38.6	9	3.0	1	10
0.48	"	2	9	50.8	9	4.4	0	0
0.96	"	2	10	33.8	10	4.1	0	0
..	"	4	21	8.9	7	2.7	14	66.6
0.06	"	4	20	7.1	4	4.7	16	80
0.12	"	4	21	11.1	14	2.8	7	33.3
0.24	"	4	9	10.2	6	6.1	3	33.3
0.48	"	4	10	11.7	8	2.3	2	20
0.96	"	4	9	15.1	8	5.2	1	11.1

TABLE III.—THE EFFECT OF HYDERGINE AND CERTAIN ANALGETIC DRUGS ON THE PERCENTAGE OF RABBITS FAILING TO DEFECATE AFTER DRUG INJECTION

Hydergine Dose, mg./Kg.	Morphine or *l*-Isomethadone Dose, mg./Kg.		No. of Observations Made	Rabbits Failing to Pass Scybala After Injection, % 0–1 Hr.	1–3 Hr.	3–5 Hr.	5–7 Hr.
..	(Saline)	1 ml.	287	21	3	1	1
0.03	..	..	16	18.7	12.5	0	0
0.06	..	..	15	20	20	6.6	0
0.12	..	..	16	31	6	19	0
..	Morphine	1	32	37	18	3	3
0.06	"	1	16	50	75	31	31
..	"	2	32	56	50	34	3
0.06	"	2	16	25	81	87	25
..	*l*-Isomethadone	1	16	25	37	6	0
0.06	"	1	16	25	43	25	12.5
..	"	2	16	37	37	25	0
..	Acetylmethadol	0.5	16	93	62	75	0
0.06	"	0.5	16	37	87	62	56
..	"	1	15	33	86	53	26
..	*n*-Allylnormorphine	2	16	75	25	6	0
0.06	" "	2	16	81	50	12	19

plained as due to the stimulatory action of 0.12 mg./Kg. of Hydergine® to provide an immediate defecatory response in some rabbits while inhibiting defecation in a larger percentage than in the control group. Minor differences in the two methods are seen also when Hydergine® is injected before morphine. The percentage of nondefecating rabbits following 2 mg./Kg. of morphine increased from 21% (saline control) to 56% while prior administration of 0.06 mg./Kg. of Hydergine® reduced this to 25%. Apparently fewer rabbits "failed to defecate" when Hydergine® preceded morphine due to prompt defecation. Figure 2 shows this more clearly where it is seen that a high count resulted for the first hour. After the first hour closer correlation of the data obtained by these two methods is noted. Comparison of the data in Table III with Figs. 1 to 4 shows that not only are the larger doses of Hydergine constipating but that this drug enhances the constipative effects of 2 mg./Kg. of morphine, 1

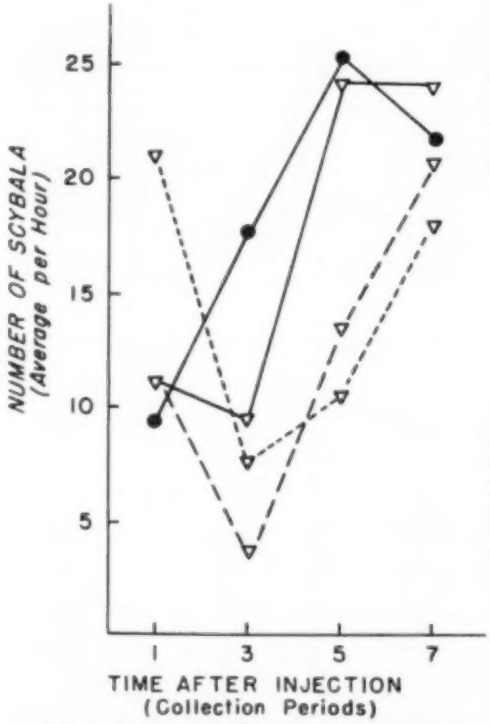

Fig. 1.—Effect of Hydergine on intestinal motility of the rabbit using Eddy's method and counting the average number of scybala passed during each observation period. Δ——Δ, Hydergine, 0.03 mg./Kg.; ●——●, control; Δ— — —Δ, Hydergine, 0.06 mg./Kg.; Δ– – –Δ, Hydergine, 0.12 mg./Kg.

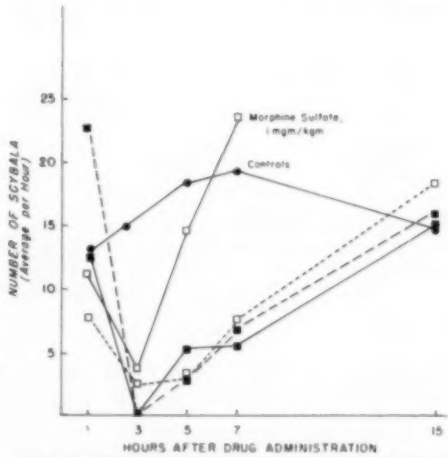

Fig. 2.—Effect of morphine and Hydergine given prior to morphine on intestinal motility in rabbits. □——□, morphine sulfate, 2 mg./Kg.; ●——●, controls; ■– – –■, morphine sulfate, 2 mg./Kg. and Hydergine, 0.06 mg./Kg. ■——■, morphine sulfate, 1 mg./Kg. and Hydergine, 0.06 mg./Kg.

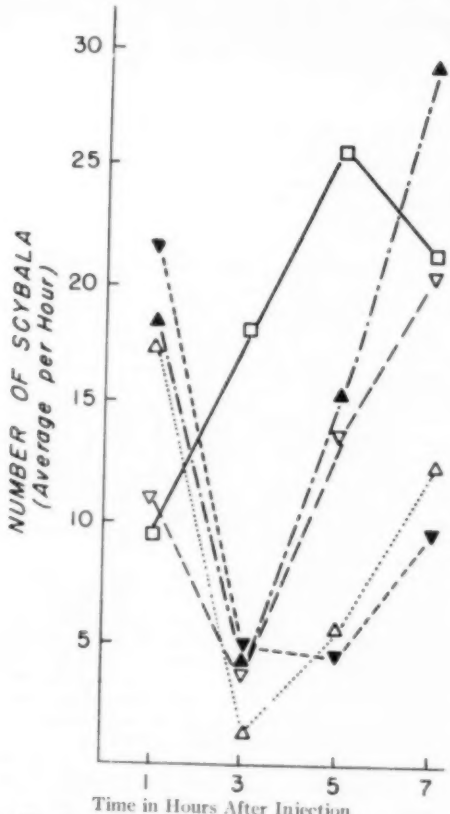

Fig. 3.—Effect of prior administration of Hydergine on changes in intestinal activity in the rabbit caused by *l*-isomethadone. ▲——▲ *l*-isomethadone, 1 mg./Kg.; □——□ control; ▽----▽ Hydergine, 0.06 mg./Kg.; Δ-------Δ Hydergine, 0.06 mg./Kg. and *l*-isomethadone, 1 mg./Kg.; ▼----▼ *l*-isomethadone, 2 mg./Kg.

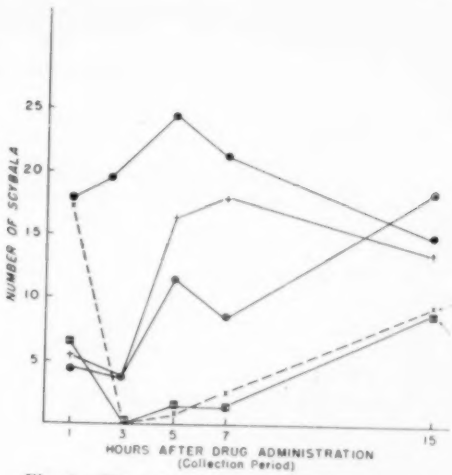

Fig. 4.—The potentiation of intestinal effects of N-allylnormorphine and α-acetyl methadol by Hydergine in rabbits. ⊕——⊕, *n*-allylnormorphine, 2 mg./Kg. and Hydergine, 0.06 mg./Kg.; ●——●, control; +——+, *n*-allylnormorphine, 4 mg./Kg.; ×----×, alpha acetyl methadol, 1 mg./Kg.; ×——×, alpha acetyl methadol, 0.5 mg./Kg. and Hydergine, 0.06 mg./Kg.

mg./Kg. of *l*-isomethadone, 0.5 mg./Kg. of *dl*-alpha acetylmethadol, and 2 mg./Kg. of *n*-allylnormorphine.

DISCUSSION

Hydergine® depressed intestinal activity slightly in both the rat and rabbit. When administered prior to analgetics there was increased constipation. This effect was especially evident in the increased incidence of pylorospasms seen in the rat when 0.06 mg./Kg. of Hydergine® preceded either the 2 or 4 mg./Kg. dose of *l*-isomethadone. With higher doses of Hydergine®, however, there was a slight decrease in the intense constipation caused by *l*-isomethadone. Similarly, the dose of 0.06 mg./Kg. of Hydergine® in the rabbit increased the constipation caused by the several analgetics used.

The constipative effect of the 0.06 mg./Kg. dose of Hydergine® used alone or with analgetic drugs is difficult to explain. Recalling Sato's (10) demonstration that morphine did not cause constipation in the sympathectomized rabbit, it would be expected if 0.06 mg./Kg. of Hydergine® is adrenolytic, a decrease in morphine-induced constipation should result. Rothlin (11) claims that Hydergine® in the low dose of 0.06 mg./Kg. is not adrenolytic. Possibly the explanation of why the dose of 0.06 mg./Kg. of Hydergine® is slightly constipating when used alone or why it potentiates morphine constipation lies in a central effect on the autonomic centers. While there is some evidence that this dose of Hydergine® potentiates the depressant effects of the barbiturates (12) through central action on "vegetative brain centers" (13), the effect here is probably one of stimulation of the sympathetic (adrenergic) centers. The immediate defecatory response sometimes observed in rabbits when Hydergine® is injected also could be due to a central effect on the autonomic centers.

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Synthetic Laxative Drugs*

By N. M. FERGUSON

Data are presented on the laxative effects produced in mice by the administration of several synthetic polyhydroxyanthraquinone glycosides and their aglycones. These compounds are compared in laxative activity to standard senna powder using the fecal staining technique. Through the addition of a mixture of potassium hydroxide and magnesium hydroxide, the laxative activity of some of the synthetic glycosides is greatly increased.

LAXATIVE DRUGS and their constituents, particularly those of the polyhydroxyanthraquinone type, have been under investigation for many years. The work of Léger (1–3), Rosenthaler (4, 5), Beal and his co-workers (6, 7), Fairbairn (8, 9), Stoll (10, 11), and many others centered around the isolation and identification of the polyhydroxyanthraquinones and their corresponding glycosides from aloe, cascara, frangula, rhubarb, and senna. As a result of these investigations, the aglycones present in the the above drugs were isolated and identified. Their glycosides, however, could not be purified and identified due to the ease of hydrolysis and the difficulties involved in separation. Because of these facts, no plant substance of this type which produces laxation in man or animals has been isolated, and its structure established.

The difficulties involved in the identification of plant glycosides led Gardner and his co-workers (12–17), to the synthesis of several polyhydroxyanthraquinone glycosides in an attempt to duplicate the chemical properties of the natural glycosides and, thereby, effect an identification of these natural products.

The purpose of this research is to study the laxative effect in mice produced by the administration of certain of these synthetic polyhydroxyanthraquinone glycosides.

EXPERIMENTAL

The glycosides used in the present study were synthesized using the method of Takahashi (18). This method consists of reacting the appropriate aglycone with the desired bromo-acetylated sugar in the presence of quinoline and dry silver oxide. The acetylated glycoside is then separated and purified and the pure glycoside obtained from it by deacetylation. The preparation of some of the glycosides used in this study is described elsewhere (19), the remainder will be described in a later paper.

The laxative testing technique used was the mouse fecal staining method described by Hazelton (20), and by Lou (21), except that free access to food and water was provided at all times during the test. Female albino mice, weighing 20 to 25 grams which had been acclimated to the laboratory for two weeks prior to the test, were placed in small cages protected from drafts and provided with food[1] and water and observed for fecal stains for thirty minutes before dosing. The tendency to stain was determined by examining the lower surface of Whatman #1 filter paper placed beneath each cage. Mice producing stools which were moist enough to produce a fecal stain through the filter paper were discarded.

The remaining mice were then dosed by stomach tube with a single dose of the laxative preparation dissolved or suspended in 0.5 cc. of the dosing solution. The stomach tube consisted of a blunted spinal puncture needle attached to a syringe. The nature of the dosing solution and the results obtained are shown in Table I. Readings were taken every two hours for twelve hours and mice which produced a stain at any time during this period were recorded as positive. It was found that the mice could be re-employed in the test after a rest period of one week.

Since mice are very susceptible to air currents, and changes in room temperatures, they often become stainers if the room temperature drops below 25°. In order to control these two factors, individual treatment cages made by fitting a hinged lid to wire test tube baskets 12.5 cm. × 10 cm. × 10 cm. and fitted with water drinking tubes were used. Each set of 10 cages was covered with a cardboard carton equipped with an electric light bulb and small ventilator holes. In this way, it was possible to maintain the proper minimum temperature and at the same time keep the laboratory drafts from chilling the mice.

The dosing solutions used were water, 0.4% sodium carbonate solution, and a suspension of magnesium hydroxide in a dilute solution of potassium hydroxide. The sodium carbonate solution was used in order to solubilize the drugs being tested. The magnesium hydroxide–potassium hydroxide solution was chosen first in order to solubilize the drugs used and second because of the fact that ash determinations revealed the presence of magnesium oxide in senna leaves, as well as in frangula bark, and cascara bark.

In order to determine what effect a change in the concentration of magnesium hydroxide and potassium hydroxide in the dosing solution would have

* Received April 13, 1956, from the Pharmacognosy Research Laboratory, University of Houston, College of Pharmacy, Houston, Texas.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April, 1956.
The author wishes to thank Dr. Chen and his associates at Eli Lilly and Company for running certain of the assays as well as for many helpful suggestions.

[1] Ralston Purina Checker Tabs.

TABLE I.—THE LAXATIVE EFFECT IN MICE PRODUCED BY SEVERAL ANTHRAQUINONE DERIVATIVES AND THEIR CORRESPONDING GLYCOSIDES COMPARED WITH THAT PRODUCED BY ALOE AND SENNA

		Dosing Solution (per 0.5 ml. Dose)					
		Distilled Water		$NaHCO_3$ Solution 1.88×10^{-4} Moles		$Mg(OH)_2$ Solution 14.2×10^{-7} Moles KOH Solution 23.3×10^{-4} Moles	
Drug	Drug Dose, mg.	Number Treated	Per Cent Positive	Number Treated	Per Cent Positive	Number Treated	Per Cent Positive
Senna-Like Stools							
Dosing solution	...	10	0	10	0	20	0
Sodium chloride	0.016	10	0	..	...	10	0
Standard senna powder	6.25	40	50	..	...	..	...
Chrysazin	20.0	20	0	..	...	20	0
Chrysazin	5.0	20	0	..	...	..	...
Chrysazin	0.128	20	0	..	..	..	...
Chrysazin glucoside	5.0	10	50	10	50	..	...
Chrysazin glucoside	2.5	10	10	20	50	..	...
K chrysazin glucoside	2.56	20	75	..	...	10	100
K chrysazin glucoside	1.28	20	40	..	...	10	100
K chrysazin glucoside	0.64	10	10	..	...	10	100
K chrysazin glucoside	0.128	..	...	..	...	10	100
K chrysazin glucoside	0.064	..	...	..	...	20	65
Rhein	0.128	10	0	..	...	10	0
Rhein glucoside	0.141	10	0	..	...	..	...
K rhein glucoside	0.141	10	10	..	...	20	60
K rhein glucoside	0.070	10	0	..	...	10	60
Chrysophanic acid	5.0	10	20	..	...	10	20
Chrysophanic acid glucoside	4.6	10	20	30	43	..	...
Chrysophanic acid glucoside	2.3	10	0	10	20	..	...
Chrysophanic acid acetyl glucoside	2.3	10	0	10	10	..	...
Chrysophanic acid maltoside	6.5	10	30	10	100	..	...
Chrysophanic acid maltoside	5.0	10	20	..	...	..	...
Chrysophanic acid acetyl maltoside	6.5	10	0	10	0	..	...
K chrysophanic acid anthranol-9-glucoside	2.47	10	0	..	...	..	...
K chrysophanic acid anthranol-9-glucoside	0.128	10	0	..	...	10	60
Aloe-Like Stools							
Powdered extract aloes N. F. VII	40.0	25	77	..	...	..	...
Powdered extract aloes N. F. VII[a]	30.0	50	58	..	...	..	...
Powdered extract aloes N. F. VII[a]	20.0	93	54	..	...	..	...
Powdered extract aloes N. F. VII[a]	15.0	29	45	..	...	..	...
Aloin U. S. P. XIV	20.0	10	40	..	...	..	...
Aloin U. S. P. XIV	7.5	10	10	10	20	..	...
Aloin U. S. P. XIV	5.0	10	10	10	20	..	...
Aloe emodin	10.0	10	10	10	40	..	...
Aloe emodin	5.0	10	0	10	0	..	...
Aloe emodin	3.15	10	0	10	0	..	...
Aloe emodin glucoside	7.5	10	70	10	100	..	...
Aloe emodin glucoside	5.0	..	...	10	80	..	...
Aloe emodin glucoside	2.0	..	...	10	10	..	...
Aloe emodin maltoside	5.0	10	80	10	100	..	...
K aloe emodin maltoside	1.0	8	0	10	10	..	...
Frangula emodin	10.0	10	0	10	10	..	...
Frangula emodin	5.0	10	0	10	0	..	...

Aloe-Like Stools

Drug	Drug Dose, mg.	Dosing Solution (per 0.5 ml. Dose) — Distilled Water — Number Treated	Distilled Water — Per Cent Positive	Na_2CO_3 Solution 1.88×10^{-3} Moles — Number Treated	Na_2CO_3 Solution 1.88×10^{-3} Moles — Per Cent Positive	$Mg(OH)_2$ Solution 14.2×10^{-3} Moles KOH Solution 23.3×10^{-3} Moles — Number Treated	$Mg(OH)_2$ Solution 14.2×10^{-3} Moles KOH Solution 23.3×10^{-3} Moles — Per Cent Positive
Frangula emodin glucoside	5.0	15	66	..	...	..	...
K frangula emodin glucoside	0.75	8	0	..	...	..	...
Frangula emodin maltoside	6.5	10	50	10	70	..	...
Frangula emodin maltoside	4.0	12	30	..	...	..	...
Frangula emodin maltoside	2.0	10	10	..	...	..	...
K frangula emodin maltoside	1.0	10	100	..	...	..	...
Alizarin-2-glucoside	8.4	10	20	10	40	..	...
Alizarin-2-glucoside	4.2	10	10	10	20	..	...
K alizarin-2-glucoside	2.4	10	50	..	...	..	...
Hydroxy-chrysazin-2-glucoside	5.0	..	...	10	70	..	...
K hydroxy-chrysazin-2-glucoside	0.128	..	...	..	...	10	20

[a] See reference (20).

on the laxative action of potassium chrysazin glucoside several tests were run in which the concentration of these substances was varied. The results of these tests are shown in Table II. Additional tests in which calcium hydroxide is substituted mole-for-mole for the magnesium hydroxide in the above dosing solution and another in which aluminum hydroxide in equimolar concentration is substituted for the magnesium hydroxide are shown in Table III.

In order to rule out the salt effect of the compounds tested a group of 10 mice were each dosed with 0.016 mg. of sodium chloride in water and again in the magnesium hydroxide–potassium hydroxide dosing solution. Here the amount of sodium chloride used is equivalent in number of particles to 0.128 mg. of potassium chrysazin glucoside.

TABLE II.—THE EFFECT OF CHANGING THE CONCENTRATION OF POTASSIUM HYDROXIDE AND MAGNESIUM HYDROXIDE ON THE LAXATIVE ACTION OF POTASSIUM CHRYSAZIN GLUCOSIDE 0.128 MG. (1.45×10^{-7} MOLES) PER DOSE OF 0.5 ML. IN MICE

Senna-Like Stools

Dosing Solution Concentration: Moles KOH $\times 10^{-3}$	Moles $Mg(OH)_2$ $\times 10^{-7}$	Number of Animals Treated	Per Cent Positive
23.30	...	20	15
23.30	1.42	20	10
23.30	2.84	10	10
23.30	5.64	10	30
23.30	8.46	10	40
23.30	14.20	10	100
23.30	71.00	10	100
...	14.20	20	25
4.66	14.20	10	20
14.10	14.20	10	40
23.30	14.20	10	100
18.60	71.00	10	50

RESULTS AND DISCUSSION

In observing the laxative effects in mice of senna and aloe preparations, as well as those of the synthetic aglycones and glycosides reported here, it was apparent from the beginning that two distinct types of laxative activity were possible. The first which we will refer to as the senna type, is produced by senna and its preparations as well as by all of the glycosides investigated having the sugar component attached in the 1 or 8 position in such anthraquinone derivatives that have no hydroxy groups in positions 2, 3, 6, or 7. The presence of a carboxy or methyl group in position number 3 such as is the case in rhein or chrysophanic acid does not change the senna-like properties of the glycosides prepared from these aglycones. The administration of senna-like drugs causes the mice to produce stools which are soft and watery and show distinct stains when collected on filter paper. Such stools are sufficiently moist to enable the stains to be seen from the underneath side of Whatman #1 filter paper. Aside from the number of stains observed, the extent of staining was also found to be important since reduced dosage of drug does not always cause a decrease in the number of mice producing stains but only in the intensity of staining.

TABLE III.—A COMPARISON OF THE LAXATIVE EFFECTS PRODUCED BY POTASSIUM CHRYSAZIN GLUCOSIDE IN POTASSIUM HYDROXIDE SOLUTION IN THE PRESENCE OF MAGNESIUM HYDROXIDE, CALCIUM HYDROXIDE, AND ALUMINUM HYDROXIDE IN MICE

Dosing Solution Concentration: Moles KOH $\times 10^{-3}$		Number of Animals Treated	Per cent Positive
	Moles $Ca(OH)_2$ $\times 10^{-7}$		
23.3	14.2	19	35
..	Moles $Al(OH)_3$ $\times 10^{-7}$	..	...
23.3	14.2	20	30
..	Moles $Mg(OH)_2$ $\times 10^{-7}$	..	...
23.3	14.2	10	100

The second type of laxative activity is that displayed by aloes and aloin as well as by aglycones and

their glycosides having hydroxy groups in positions 2, 3, 6, or 7. The aloe-like action results in larger, shinier, more pasty stools which will adhere to the filter paper but will not produce distinct stains. Because of this fact, it is difficult in low concentrations to differentiate between the stools obtained from normal as against those obtained from treated animals. Anthraquinone derivative aglycones such as aloe emodin, frangula emodin, alizarin and hydroxychrysazin, as well as preparations of cascara, frangula, and rhubarb, produce an aloe-like laxative action in mice.

It can be seen from the data reported in Table I, that standard senna powder is 50% active in doses of 6.25 mg. whereas powdered extract of aloe must be given in doses of 20 mg. to produce the same activity. It is further seen, that aloin in doses of 20 mg. produces laxation in only 40% of the mice treated. The aglycones such as chrysazin, rhein, chrysophanic acid, aloe emodin, and frangula emodin, are either inactive or only slightly active under the same conditions.

The glycosides prepared from the aglycones mentioned above show increased activity over the corresponding aglycones when given in water. When suspended in sodium carbonate solution, these glycosides show a marked increase in activity due probably to increased solubility and uniformity of dosage.

The use of the magnesium hydroxide–potassium hydroxide dosing solution had no effect on the activity of the aglycones such as chrysazin, rhein, or chrysophanic acid over that produced when these compounds were administered in water. This was not the case with the glycosides, however. It can be seen from Table I, that potassium chrysazin glucoside given in doses of 2.56 mg. in aqueous solution gave a positive response of 75%. The same effect, however, was produced by only 0.064 mg. of this glucosidal salt when administered in the magnesium hydroxide–potassium hydroxide solution showing that this solution produced a definite increase in activity. Similarly, potassium rhein glucoside in doses of 0.070 mg. is inactive when administered in aqueous solution but produces 60% positive responses in the magnesium hydroxide–potassium hydroxide medium.

Potassium chrysophanic acid anthranol-9-glucoside was also prepared and tested since it has been suggested that the active constituents of aloe as well as those of senna might be anthranol glycosides. It is seen that this compound is moderately active in the magnesium hydroxide–potassium hydroxide dosing solution.

The effect of changing the potassium hydroxide as well as the magnesium hydroxide concentrations are shown in Table II. Here it is seen that the optimum concentration for potassium hydroxide is of the order of 23.30×10^{-6} moles per dose of 0.128 mg. (1.45×10^{-7} moles) of potassium chrysazin glucoside. The optimum concentration for magnesium hydroxide under the same conditions is 14.20×10^{-7} moles.

Substituting an equal amount of calcium hydroxide or aluminum hydroxide for magnesium hydroxide resulted in a decrease in the activity of potassium chrysazin glucoside. These data are presented in Table III.

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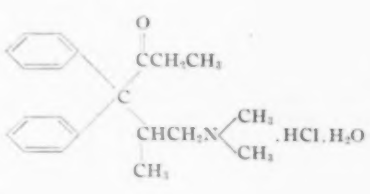
A Note on the Crystallography of *l*-Isomethadone Hydrochloride Hydrate*

By HARRY A. ROSE and ANN VAN CAMP

l-ISOMETHADONE is referred to in the literature as a narcotic and analgetic (1). Data are presented here which permit the identification of this compound by crystallographic methods.

The structural formula of *l*-isomethadone hydrochloride hydrate is as follows:



* Received April 14, 1956, from the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis 6, Ind.

TABLE I.—X-RAY DIFFRACTION DATA FOR *l*-ISOMETHADONE HYDROCHLORIDE HYDRATE

Unit cell dimensions	a_o = 13.05Å, b_o = 13.30Å, c_o = 11.75Å
Formula weights per cell	4
Formula weight	363.92
Density	1.193 Gm./cc. (flotation), 1.187 Gm./cc. (X-ray)
Axial ratio	$a:b:c$ = 0.9812:1:0.8835
Space group	$P22_12_1$

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA FOR *l*-ISOMETHADONE HYDROCHLORIDE HYDRATE

Refractive Indexes	(5893 Å, 25°). α = 1.590, β = 1.602, γ = 1.638
Optic axial angle	(+) $2V$ = 59°2′ (calcd. from α, β and γ)
Optic axial plane	001
Acute bisectrix	$\gamma = b$

TABLE III.—X RAY POWDER DIFFRACTION DATA FOR *l*-ISOMETHADONE HYDROCHLORIDE HYDRATE

d	I/I_1	$h\ k\ l$	d (calcd.)
13.38	0.08	100	13.05
9.40	0.16	110	9.32
8.58	0.08	101	8.73
7.30	1.00	111	7.30
6.65	0.20	020	6.65
5.84	1.00	002, 210	5.87, 5.86
5.32	0.60	102	5.36
4.97	0.04	112	4.97
4.66	0.08	220	4.66
4.34	0.40	202, 300, 221	4.36, 4.35, 4.33
4.16	0.40	212, 310	4.15, 4.13
3.91	0.12	311	3.90
3.64	0.40	222, 320, 113	3.65, 3.64, 3.61
3.51	0.12	302	3.50
3.35	0.32	312, 023, 040	3.38, 3.37, 3.33
3.22	0.08	140	3.22
3.09	0.20	141, 330, 322	3.11, 3.11, 3.09
3.00	0.08	331	3.00
2.86	0.16	313	2.84
2.80	0.04	114	2.80
2.66	0.08	323	2.67
2.61	0.08	500	2.61
2.49	0.04	..	..
2.40	0.08	..	..
2.35	0.08	..	..
2.28	0.08	..	..
2.17	0.04	..	..
2.07	0.04	..	..
1.959	0.08	..	..
1.877	0.04	..	..
1.769	0.04	..	..

EXPERIMENTAL

The crystals are orthorhombic prisms elongated parallel to the c axis and lying on the 100 face.

The X-ray powder diffraction data were obtained using copper radiation and nickel filter with a camera 114.6 mm. in diameter. A wave-length value of 1.5405Å was used in the calculations. The indexing of the powder pattern was done on the basis of single crystal rotation patterns around both the b and c axes.

TABLE IV.—X-RAY POWDER DIFFRACTION DATA FOR ANHYDROUS *l*-ISOMETHADONE HYDROCHLORIDE

d	I/I_1
9.20	0.03
7.96	0.53
7.18	1.00
6.53	0.67[b]
5.84	0.13
5.00	1.00
4.73	0.53
4.43	0.07
4.28	0.07
3.87	0.53[b]
3.60	0.03
3.47	0.20
3.35	0.20
3.23	0.20
3.17	0.20
3.00	0.33
2.85	0.03
2.68	0.07
2.62	0.03
2.48	0.03
2.24	0.07
2.20	0.07
2.16	0.07
2.08	0.07

The space group determination as $P22_12_1$ is uniquely determined by Weissenberg photographs in combination with the rotation photographs. These patterns show no systematic absences for ($h\ k\ l$) reflections or ($h\ o\ o$) reflections and that ($o\ k\ o$) is present only with $k = 2n$ and ($o\ o\ l$) is present only with $l = 2n$. The compound does show a positive piezoelectric effect.

On heating, *l*-isomethadone hydrochloride hydrate starts melting at 137°. However, as the temperature rises new crystals which have been identified as the anhydrous material, appear in the melt and the final melting point is 229–232°. The melt does not crystallize on cooling.

The sample of anhydrous crystals used for the powder diffraction pattern was obtained by heating the hydrate to drive off the water.

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A Note on the Preliminary Studies of Certain Pharmacological Actions of *Acorus calamus* L.*

By SOHAN L. AGARWAL, P. C. DANDIYA, K. P. SINGH, and R. B. ARORA

The alcoholic extract of *Acorus calamus* roots and rhizomes was screened for pharmacological activities. It was found to possess sedative and analgetic properties and caused a moderate depression in the blood pressure and respiration. The water-soluble fraction of the de-alcoholized extract relaxed the intestine and caused negative inotropic action on frog's heart. Anti-epileptic activity was absent.

Acorus calamus L. (1) is an aromatic herb growing throughout India and Ceylon, in marshes, wild or cultivated. The common Indian names are Ugargandha (Sanskrit), Gandhilowaj (Gujerati), Bach (Hindi and Urdu), and Lubigan (Tamil).

The roots and rhizomes of this plant are extensively used in the ayurvedic system of medicine as an emetic, laxative, carminative, diuretic, and the drug is employed in epilepsy, delirium, hysteria, and loss of memory (1). It is also used in remittent fevers and ague by the native doctors and is held in high esteem as an *insectifuge* especially for fleas. Lately, the powder of roots and rhizomes of *Acorus calamus* has been used along with other indigenous drug powders like *Rauwolfia serpentina* Benth. and has been claimed to be of value in neurosis, insomnia, melancholia, hysteria, and also indicated in neurasthenia and epilepsy (2). Rao, Sudborough, and Watson (3) have reported the presence of 1.5% essential oil in the dried roots.

The present preliminary study was undertaken to screen the extract of ugargandha roots and rhizomes for the more significant pharmacological activities that have been reported.

Two kilograms of 80-mesh powder of roots and rhizomes of the plant was extracted with hot 95% ethyl alcohol by continuous extraction process. The alcoholic extract (2 liters) was concentrated in vacuum, but all the alcohol was not removed as previous small-scale experiments had shown that the clarity of the extract was adversely affected by complete elimination of alcohol. The concentrated extract (320 ml.) was allowed to stand overnight. About 100 ml. of a dark brown oily liquid settled which was separated and reserved for future investigations. The remaining alcoholic extract was diluted to 400 ml. with 20% ethanol so that each 0.1 ml. of the alcoholic extract represented 500 mg. of the crude drug. This preparation which contained about 35% alcohol was used in most of the pharmacological experiments and will be referred as the "prepared alcoholic extract."

EXPERIMENTAL

Twenty milliliters of the "prepared alcoholic extract" was evaporated almost to dryness at 100°. The residue weighing 0.65 Gm. was shaken with 100 ml. of distilled water and the suspended matter was removed by centrifugation. A few droplets of oily liqnid floating on the surface were removed with petroleum ether. Each 0.1 ml. of this de-alcoholized alcoholic extract represented 100 mg. of the crude drug. This extract was used in the experiments on smooth and cardiac muscles and will be referred to as the "prepared de-alcoholized extract."

Toxicity.—Acute toxicity was determined in dogs and mice. Doses as high as 3 ml./Kg. of the "prepared alcoholic extract" by intraperitoneal route were tolerated by mice without any evidence of toxic symptoms. In dogs, even higher doses, up to 4 ml./Kg. were tolerated by intravenous route. The only symptom noticed in these animals was calmness, drowsiness, and mild hypnosis from which the animals could be aroused by external stimuli. However, 4 ml./Kg. in mice by the intraperitoneal route was fatal in most cases within one to two hours. These animals showed profound hypnosis and marked respiratory depression with terminal clonic convulsions resulting in death. No effort was made to determine the LD_{50}.

The same quantity of the solvent (35% ethyl alcohol) in control animals had no significant effect, the only symptom noticed being mild transient sedation as compared to the test animals.

Sedation.—The sedative property was studied in dogs, monkeys, mice, and rats. The "prepared alcoholic extract" in doses ranging from 0.4 ml. to 1.2 ml./Kg. intramuscularly caused calmness, mild sedation, drowsiness, and a tendency to sleep. The drug further showed potentiation of the hypnosis effect of pentobarbital sodium in albino rats. Thirty milligrams per kilogram of pentobarbital sodium given by intraperitoneal route in rats caused mild hypnosis in only 3 out of 10 animals and duration of hypnosis in these animals was on an average twenty minutes. The animals pretreated with 0.4 ml./Kg. of "prepared alcoholic extract," followed after a half-hour interval with pentobarbital sodium, however, all went to sleep and the average duration of hypnosis in these animals was thirty-three minutes.

Analgesia.—The hot wire method utilizing the albino rats was used to determine the analgetic activity. The tail of the animal was placed in a special brass metal holder which was cooled by running water at body temperature. The tail was then subjected to the thermal stimuli by means of an electrically heated wire to 100 volts. The control threshold of untreated animals was 10.08 seconds. A dose of 0.2 ml./Kg. of the drug intraperitoneally raised the threshold to seventeen seconds representing an increase by 70%. The quantity of alcohol used as solvent in control animals had no effect on the threshold.

Anti-epileptic Activity.—Swinyard's technique (4) using Metrazol® shock test was employed. The "prepared alcoholic extract" was administered intraperitoneally to albino rats in doses ranging from 0.2 to 0.8 ml./Kg. Drug treated animals showed milder clonic type of convulsions and were

* Received February 29, 1956, from the Department of Pharmacology, S. M. S. Medical College, Jaipur, India.
Our thanks are due to Alarsin Pharmaceuticals (India) Ltd., Bombay, for financial assistance in carrying out this work.

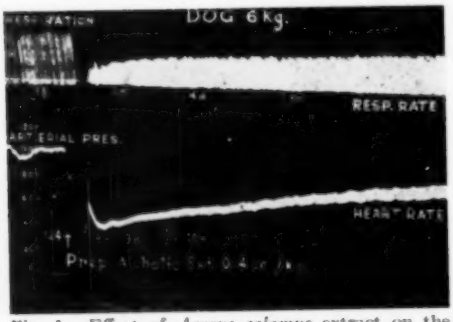

Fig. 1.—Effect of *Acorus calamus* extract on the dog's blood pressure and respiration.

without any fatalities as against severe clonic and tonic convulsions with a high mortality rate in control animals receiving Metrazol alone.

Effect on Blood Pressure.—Dogs and cats were anesthetized by intravenous administration of pentobarbital sodium (32 mg./Kg.). The carotid blood pressure was recorded kymographically by means of a mercury manometer. Two-tenths milliliter to 0.8 ml./Kg. of the "prepared alcoholic extract" was then injected into the cannulated femoral vein. A fall in blood pressure and bradycardia were produced and maintained, the recovery to pre-injection level taking place within forty-five to sixty minutes (Fig. 1). The effect varied proportionately to the doses employed.

Effect on Respiration.—The "prepared alcoholic extract" in doses ranging from 0.2 to 0.8 ml./Kg. in anesthetized dogs caused depression of respiration evidenced by transient apnea followed by diminution in the amplitude of respiratory excursions, as recorded on the kymograph by tambour connected to the trachea of the animal. However, there was marked increase in the rate of respiration (Fig. 1).

Frog Heart.—Cardiac activity was determined in *Rana tigrina* by the method of Straub (5). A

Fig. 2.—Effect of *Acorus calamus* extract on frog's heart. Upward movement of lever indicates systole.

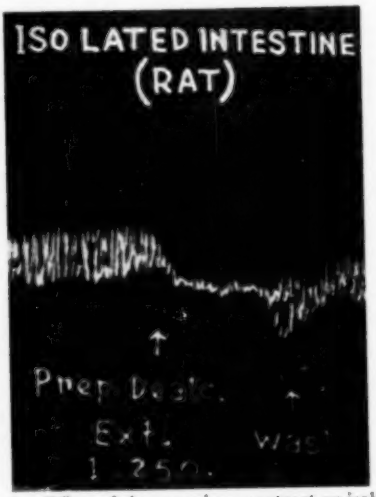

Fig. 3.—Effect of *Acorus calamus* extract on isolated rat's intestine.

1:1000 concentration of the "prepared de-alcoholized extract" in bicarbonate buffer solution slightly reduced the force of contraction of the heart while a 1:100 concentration had marked negative inotropic action (Fig. 2).

Intestine.—The effect of the "prepared de-alcoholized extract" was studied on the isolated rat's intestine employing the method of Magnus (6). Figure 3 indicates the effect of the drug in concentration of 1:250 producing a relaxation followed by normal rhythm after washing.

DISCUSSION

Action of an extract of *Acorus calamus* roots and rhizomes on experimental animals has shown the drug to possess potent sedative and analgetic properties. This justifies its historical use in the ayurvedic system of medicine for various mental diseases. The drug however did not show any significant anti-epileptic activity. The extract causes fall in blood pressure in anesthetized animals in comparatively small doses (0.2 ml. to 0.8 ml./Kg.), and produces depression of the respiration. It also causes negative inotropic action as observed in Straub heart preparation. Much higher doses than those employed in these experiments were tolerated without any evidence of toxicity in mice and dogs.

The de-alcoholized extract of the drug causes a relaxation of the smooth muscle as observed in the isolated preparation of rat's intestine.

These experiments have shown *Acorus calamus* to be a potent drug possessing significant pharmacological actions. These preliminary findings have led us to further chemical and pharmacological investigations, work on which is now in progress.

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Book Notices

Medicinal Chemistry. Vol. II. Edited by F. F. Blicke and C. M. Suter. John Wiley and Sons, Inc., New York, 1956. vi + 311 pp. 15 x 23.5 cm. Price $10.

Volume II of *Medicinal Chemistry* is the second in a series prepared under the auspices of the Division of Medicinal Chemistry of the American Chemical Society.

In general, the second volume follows the design established in the first volume. [This Journal, **40,** 363(1951)]. The present volume is composed of comprehensive reviews of four types of compounds of interest to chemists and pharmacologists. The four chapters have been written by seven specialists who are affiliated with leading research laboratories and all are well-known for their own contributions in the fields covered. The first chapter by Arthur Stoll presents "Some Chemical Aspects of the Cardiac Glycosides," supplemented by a discussion prepared by T. L. Johnson. This chapter is followed by one on "Synthetic Estrogens," prepared by John A. Hogg and Jerome Korman, of the Upjohn Company. The third section on "Analgesics and Arylpiperidine Derivatives," is the work of C. M. Suter of the Sterling-Winthrop Research Institute. The final chapter by Glenn E. Ullyot and James F. Kerwin, of the Smith, Kline, and French Laboratories, summarizes available information on "Beta-Haloethylamine Adrenergic Blocking Agents; Chemistry and Structure-Activity Relationships." Each chapter in Volume II of *Medicinal Chemistry* summarizes the biological properties, and correlates the relationship between the chemical structure and physiological activity of the type of compounds discussed. All of the compounds that have been screened for a particular type of pharmacological activity are listed within the chapter, mostly in tabular form according to the chemical group or series. Brief discussions of methods of syntheses and pharmacological testing procedures are also included in each chapter. It is planned to issue comparable volumes from time to time covering additional areas.

The present volume provides as complete a bibliography as possible for each subject covered. It includes an adequate subject index and the printing and binding are of excellent quality.

Medicinal Chemistry. Vol. III. Edited by F. F. Blicke and R. H. Cox. John Wiley and Sons, Inc., New York, 1956. vi + 346 pp. 15 x 23.5 cm. Price $10.50.

This latest volume includes four additional chapters covering important segments of the medicinal chemistry field. The first chapter is entitled "Methadone and Related Analgesics," which was contributed by Thomas P. Carney of the Lilly Research Laboratories. Here the chemistry of methadone, isomethadone, and related compounds is summarized based on the literature through 1952. The distribution and metabolism of methadone and the pharmacological activity of compounds coming in this category are also summarized. The second chapter, written by Peter L. deBenneville, of Rohm and Haas Company, is devoted to the quaternary ammonium germicides. The "Non-Mercurial Diuretics," by Victor Papesch and Elmer F. Schroeder, of G. D. Searle and Company, constitutes the third chapter. The subjects discussed in this chapter include the measurement of diuretic activity and the mode of action of the nonmercurial diuretics. The main subject is divided into osmotic diuretics; xanthines; pyrimidines, imidazoles, and triazines; ureas, amides, and amines; miscellaneous organic compounds; cation exchange resins; plant extracts with diuretic activity; and hormones. In the fourth chapter, contributed by Arthur Stempel and John A. Aeschlimann, of Hoffmann-LaRoche, Inc., pertinent information on synthetic analogs of physostigmine is presented. Screening procedures for these compounds are described, correlation of structure and activity is reviewed, and clinical applications and modes of action are also covered.

New and Nonofficial Remedies 1956. Issued under the direction of the Council on Pharmacy and Chemistry of the American Medical Association. J. B. Lippincott Company, Philadelphia, Pa. xxv + 540 pp. 13 x 19.5 cm. Price $3.35.

The 1956 edition of this important annual publication of the American Medical Association, prepared under the auspices of its Council on Pharmacy and Chemistry, has been written in accordance with a revised program for the evaluation of drugs, which was instituted in 1955. It is composed of monographs on many new drugs and includes available evidence which may or may not be adequate to determine their future value as therapeutic agents. Relationship of new drugs to older and well-established drugs has been emphasized in some instances. The listing of a drug in *New and Nonofficial Remedies* no longer implies approval by the Council on Pharmacy and Chemistry. Drugs included in the new edition are evaluated objectively, but the reader must draw his own conclusions as to how this information should be interpreted. Technical information about dosage forms is no longer given and names of manufacturers and distributors are omitted. Nonproprietary names are used for all drugs, but nearly all available trademark names under which preparations of such drugs are marketed are included. Monograph descriptions of mixtures of drugs have been discontinued in order to enable the Council to give more attention to the

evaluation of evidence relative to the actions, uses, dosage, hazards, and other significant properties of individual drugs. The Council apparently believes that prompt publication of significant information on each new basic drug will make it possible for the physician to judge what combinations are desirable in specific cases.

NNR 1956 contains 58 new monographs on drugs not previously described. In the revision of the 1955 edition, seven monographs were omitted for drugs which are considered well-known because of their inclusion in NNR, U. S. P., or N. F. for the accumulative period of twenty years or which are no longer available. *New and Nonofficial Remedies* for many years has been an important reference book for the medical and pharmaceutical professions. The fact that certain information relating to dosage forms and names of distributors has been omitted may impair its value as a reference book for pharmacists, but this information is readily available elsewhere. *NNR 1956* should be a part of the library of every pharmacy where authentic and unbiased information about the therapeutic value of new drugs is needed.

Principles of Fungicidal Action. (Vol. 30 of "New Series of Plant Science Books.") The Chronica Botanica Company, Waltham, Mass., and Hafner Publishing Company New York City, 1956. xxiii + 279 pp. 15.5 x 23.5 cm. Price $3.50.

This book is a compilation of available information on mechanisms of fungicidal action, with distinctions between permeation and inherent toxicity. The text opens with a chronological listing of important events in fungicidal history from 1000 B.C. to 1952 and a brief introduction. This is followed by chapters on: fungicidal action and its measurement, protection and its measurement, mobilization of a residual protectant, permeation into the fungus, disruption of cellular organization, effect on mitosis, morphology and growth, effect on metabolism of the fungus, chelation of needed metals, the action of metals, the action of sulfur, action of organic sulfur compounds, action of quinones and other ketones, action of heteocyclic compounds, and chemotherapy of plant diseases. A bibliography, a general index, and an author index are appended. The book should be useful to plant pathologists and it is a good reference book for those interested in the field of insecticides and fungicides.

Hormones and the Aging Process. By EARL T. ENGLE and GREGORY PINCUS. ACADEMIC PRESS INC., New York, 1956. 323 pp. 15.5 x 23.5 cm. Price $8.50.

This book is a record of a symposium on the relationship of hormones to the process of aging, which was held in May 1955. The scope of the symposium is indicated by the titles of the contributed papers: aging and urinary steroid excretion, effect of aging on the steroid metabolism as reflected in plasma levels, the thyroid in the aging process, urinary excretion of gonadotropin as a function of age, hormonal regulation of muscle development, steroids and protein metabolism in experimental animals, androgenic and anabolic action of testosterone derivatives, naturally occurring pathology in the aging rat, effects of hormones on protein metabolism, the role of steroids in calcium and phosphorus metabolism, newer techniques in the study of calcium metabolism in man and effects of hormones thereon, mechanisms regulating fluid and electrolyte metabolism, steroid hormones in osteoporosis, sex steroid replacement in the aging individual, effects of steroids in women with breast cancer, endocrine regulation of prostatic growth, the effects of some of the steroid hormones on the metabolic balances in aged males, and cerebral metabolism in the aging process: the steroid factor. The discussion after the presentation of each paper is included in the text. Appended author and subject indexes make the book a useful reference volume.

BOOKS RECEIVED

1955 Vacuum Symposium Transactions. Committee on Vacuum Techniques, Inc., Boston, Mass., 1956. vii + 101 pp. 22 x 29 cm. Price $10.

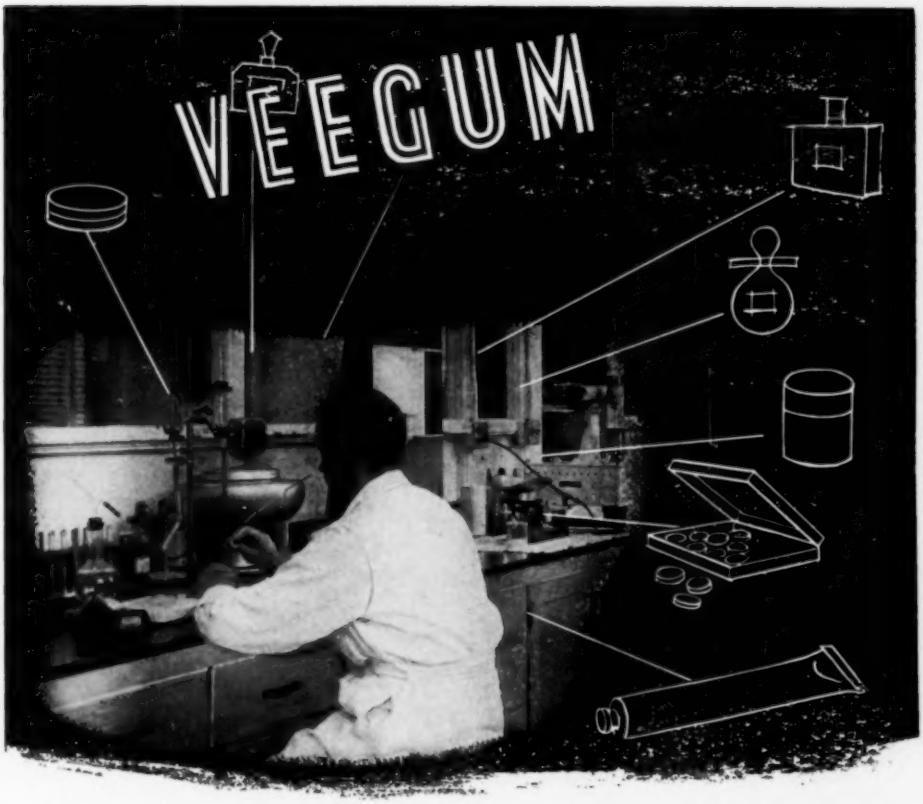
Chemical Who's Who 1956. By WINFIELD SCOTT DOWNS and WILLIAMS HAYNES. Lewis Historical Publishing Company, Inc., New York, 1956. xvi + 1267 pp. 15.5 x 23.5 cm.

Blakiston's New Gould Medical Dictionary. 2nd ed. By NORMAND L. HOERR and ARTHUR OSOL. McGraw-Hill Book Company, Inc., New York, 1956. xxvi + 1463 pp. 17.5 x 25.5 cm. Price $11.50.

The Examination of New Organic Compounds. By WALTER T. SMITH, JR., and RALPH L. SHRINER. John Wiley & Sons, Inc., New York, 1956. vii + 136 pp. 21.5 x 29 cm. Price $3.50.

Apotekens Register over Standardförpackade Läkemedel 1956. Victor Pettersons Bokindustri Aktiebolag, Stockholm, Sweden, 1956. vii + 528 pp. 18 x 23 cm.

Currents in Biochemical Research 1956. By DAVID E. GREEN. Interscience Publishers, Inc., New York, 1956. xvi + 697 pp. 16 x 28.5 cm. Price $10.



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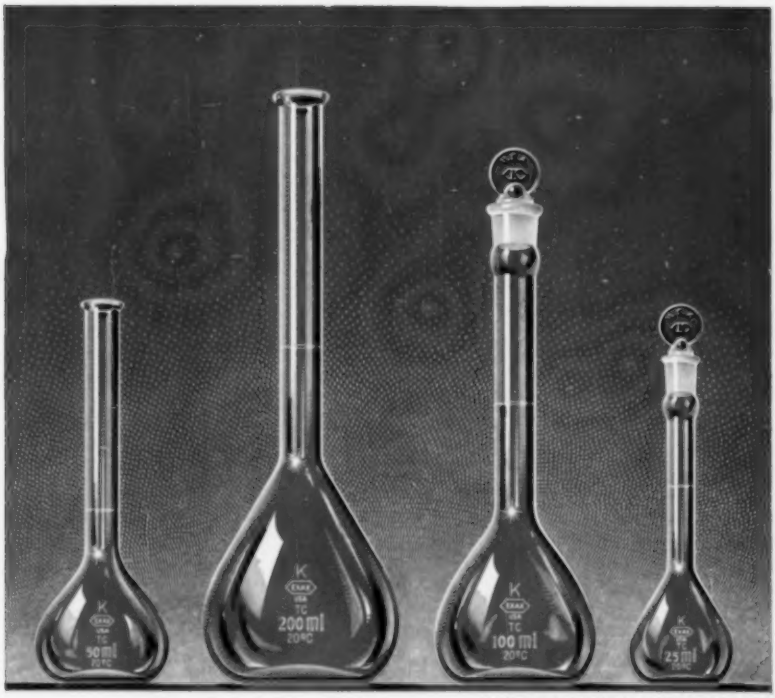

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